CENTRAL MECHANISMS IN THE CONTROL OF SEASONAL BREEDING IN THE SOAY RAM

FRANCIS JOHN PETER EBLING

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CONTENTS

DECLARATION.

Except where acknowledgement is made by reference, the experiments described in this thesis were the unaided work of the author.

No part of this work has already been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for another degree.

F. J. P. Ebling

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Abstract

The aims of the experiments described in this thesis were 1) to investigate the role of endogenous opioid peptides (EOP) in the control of pulsatile LH release in the ram, and 2) to study the generation of pineal melatonin rhythms and their role in relaying the effects of photoperiod on seasonal reproductive cycles in rams.

A series of experiments was carried out on the effects of pharmacological opiate antagonists and agonists on LH secretion. Morphine significantly reduced LH pulse frequency in sexually active rams, and this effect was reversible by concurrent administration of the opiate antagonist naloxone, thus indicating the existence of specific opiate receptors. Naloxone injections alone increased LH pulse frequency in rams, showing that EOP mechanisms inhibit tonic LH secretion in a physiological situation. Effects of naloxone were studied at different stages of the seasonal reproductive cycle in rams maintained out-of-doors. Responses in intact rams were greater in the breeding season in September and December than when the rams were sexually quiescent in March and June. In pinealectomized rams which displayed a premature and attenuated reproductive cycle, LH responses to naloxone also correlated with testicular activity. These observations support the hypothesis that EOP mechanisms mediate the inhibitory effects of negative steroid feedback on LHRH secretion in the hypothalamus.

Radioimmunoassay techniques were developed and validated for the extraction and measurement of $\beta_o$endorphin in tissue and plasma. Substantial levels of immunoreactive $\beta_o$endorphin (β_oEP) were found in the preoptic area and median eminence/arcuate nucleas area in the hypothalamus of the sexually active ram. This distribution is similar

to that previously observed for LHRH, and is consistent with the view that the effects of naloxone on LH secretion result from antagonism of inhibitory EOP mechanisms on LHRH release. A considerable seasonal variation in peripheral blood plasma β_oEP concentrations was observed, levels being 5-20 fold higher under short days than under long days. Studies suggest that this material is of pituitary origin, but its physiological function in peripheral target tissues is unknown.

Studies were also made of melatonin rhythms and sexual responses in rams housed for prolonged periods under constant illumination (LL) and constant darkness (DD). After eight weeks under DD melatonin levels were not constantly elevated to normal nocturnal levels, though no clear 24 hour periodicity was evident. Likewise, under LL melatonin levels were not constantly suppressed. DD was unable to prevent gonadal regression in rams previously housed under photostimulatory short days, thus rams do not show the phenomenon of relative photorefractoriness. Paradoxically, stimulated testicular growth occurred in rams transferred from inhibitory long days to LL.

A one hour light pulse per 24 hours re-established the melatonin rhythm in rams maintained in DD. The onset of the melatonin peak was shortly after the light pulse, suggesting that the pulse acts as an entrainment cue rather than by direct suppression of melatonin release. In a short term experiment a one hour light pulse was able to phase shift established melatonin rhythms free-running in constant darkness. A second light pulse given 7 hours after the first had only a transient suppressive effect, thus the effect of light pulses on melatonin levels may depend on their temporal relationship to the endogenous melatonin rhythm. These observations are consistent with a model in which the

environmental light-dark cycle regulates the melatonin rhythm by entraining two or more endogenous oscillators. The role of circadian rhythms in photoperiodic time measurement would appear to be in the generation of melatonin rhythms rather than in their ultimate interpretation by the hypothalamus.

Based on the current results and a survey of the relevant literature, a model is proposed in which EOP are involved in the central mechanism regulating gonadotrophin secretion in seasonal breeding mammals, and the pineal melatonin rhythm is involved in relaying the effects of photoperiod on these central mechanisms. It remains to be established whether melatonin mediates its effects by directly influencing the central EOP mechanisms.

Glossary of abbreviations.

ACTH	Adrenocorticotrophin
ANOVAR	Analysis of variance
β-EP	Immunoreactive β-endorphin
CNS	Central nervous system
DD	constant dark (= constant dim red light)
EOP	Endogenous opioid peptide(s)
FSH	Follicle stimulating hormone
H-P-G	Hypothalamic-pituitary-gonadal
i.v.	intravenous
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone
LL	Constant illumination
Long day	Photoperiod in which the length of the light phase exceeds the length of the dark phase
mg/kg b.w.	Dose expressed in milligrams per kilogram body weight
MOR	Morphine
NAL	Naloxone
Non-pineal	Denervated or removed pineal
PINx	Pinealectomized
RIA	Radioimmunoassay
SCGx	Superior cervical ganglionectomized
Short day	Photoperiod in which the length of the light phase is less than the length of the dark phase

VYCOR	Activated glass powder used for extraction of β-EP from plasma
$\mu, \delta, k, \epsilon, \sigma$	Mu, delta, kappa, epsilon, sigma : opiate receptor subclasses as defined by Wood (1982)
Skeleton photoperiod	A skeleton photoperiod is one in which discrete short periods of light convey information about daylength by means of their temporal relationship rather than by their absolute duration. For example, a skeleton photoperiod of 8L:7D:1L:8D may be interpreted as a long day even though the total hours of light (9L) would constitute a short day if given as a single continuous period of light.

Chapter 1

General introduction

1.1 Seasonal breeding and photoperiodism in sheep

The phenomenon of seasonal breeding in sheep has been extensively described (Marshall, 1937; Lincoln and Short, 1980). Considerable variation in seasonality has been observed between different breeds, those originating from more northerly latitudes tending to have a later and more pronounced breeding season than those which have evolved at lesser latitudes (Hafez, 1952). The semi-domesticated Soay breed on St. Kilda (58°N) is a good example of a strongly seasonal breed. The rams start rutting in late September and the ewes begin oestrous cycles in October. Most conceptions occur in the second oestrous cycle in November resulting in the birth of lambs the following April (Grubb and Jewell,1973). The ultimate factors such as food supply and climate which determine the survival of the young cannot directly provide a time cue to initiate the reproductive process in species which have a long gestation period. Mechanisms have therefore evolved to anticipate the optimal time for births by which reproductive activity is initiated in response to proximate factors. Such proximate factors need not necessarily have any direct effect on survival of the young (Hoffmann, 1981).

In common with many temperate mammals and birds, changes in daylength are the main proximate cue for sheep (Yeates, 1949; Follett, 1978). Sheep are often described as a "short day" species because the decreasing daily photoperiods of autumn induce testicular recrudescence in rams and onset of ovarian cyclicity in ewes, and long days induce gonadal regression (Karsch *et al.*, 1984). However if rams are maintained for prolonged periods on constant short days, spontaneous testicular regression occurs, thus rams become refractory to the stimulatory effects of short days (Lincoln, 1980; Howles *et al.*, 1982).

Likewise, rams also become insensitive to the inhibitory effects of long daylengths when maintained on them for prolonged periods. Refractoriness to photoperiod has been observed in many other species, for example in the Syrian hamster (Mesocricetus auratus). Short days initially induce gonadal regression in this species, however after prolonged exposure to short days gonadal recrudescence occurs spontaneously (Stetson and Tate-Ostroff, 1981). It has been suggested that in sheep photorefractoriness provides a mechanism to terminate the mating season, so preventing parental investment in offspring which would otherwise be born too late in the lambing season. The annual reproductive cycle of the sheep would therefore appear to consist of periods of photosensitivity interspersed with periods of photorefractoriness.

Sheep still undergo cycles of reproductive activity when kept for several years under a constant photoperiod though the amplitude of such cycles gradually decreases (rams:Howles et al., 1982; Almeida and Lincoln, 1984a; ewes: Thwaites, 1965; Ducker et al., 1973). Since the pituitary and target organs always remain capable of stimulation (rams: Lincoln, 1979c; ewes: McNeilly et al., 1982), it would appear that the central mechanisms which control the reproductive axis oscillate between active and inactive states (Turek and Campbell, 1979). The effect of photoperiod in sheep, therefore, is not to generate the annual reproductive cycle per se, but to provide an extremely precise cue to time the onset of the breeding season.

1.1.2 The role of the pineal gland and melatonin secretion

In the past two decades the importance of the pineal gland in the photoperiodic responses of mammals has been firmly established (Turek

and Campbell, 1979; Goldman and Darrow, 1983). Early evidence for a pineal involvement in reproduction was obtained in the Syrian hamster, a "long day" breeder. Animals pinealectomized while maintained on long days are unable to regress their gonads when subsequently transferred to short days (Hoffman and Reiter, 1965, 1966) or blinded (Reiter, 1967). Pinealectomy of Syrian hamsters held on short days induces premature gonadal recrudescence (Matt and Stetson, 1980; Turek and Ellis, 1981). Similar effects of pinealectomy or ganglionectomy have been observed in other long day breeders, for example in voles (Farrah and Clarke, 1976), Siberian hamsters (Hoffmann and Kuderling, 1977) and ferrets (Thorpe and Herbert, 1976). Although these results could be interpreted in terms of an anti-gonadal role for the pineal gland, in many other situations the pineal gland appears to exert an equally pro-gonadal influence. In the Turkish hamster (Mesocricetus brandti), pinealectomy of sexually active animals maintained on long days actually induces gonadal regression (Carter et al.,1982a). Pinealectomy also prevents the rapid onset of oestrous cycles in ferrets and Siberian hamsters exposed to long days (Herbert, 1981; Hoffmann and Kuderling, 1977).

In experiments of longer duration, pinealectomy may result in a total desynchrony of reproductive activity and photoperiod. Two years after pinealectomy, onset of oestrous cyclicity in female ferrets kept outdoors neither resembled the control animals nor showed any evidence of circannual periodicity (Herbert et al., 1978). Pinealectomized or ganglionectomized sheep housed indoors on artificial photoperiods are unable to display changes in reproductive and somatic activity in response to changes in photoperiod (rams: Lincoln, 1979a; Barrell and Lapwood, 1979; ewes: Bittman et al., 1983a; Brinklow and Forbes, 1984).

However, in studies where pinealectomized sheep have been kept outdoors under natural conditions, seasonal cycles of reproduction have persisted (Roche et al., 1970; Matthews et al., 1981) thus in the absence of photoperiodic information sheep may use other enviromental and social stimuli to maintain reproductive cyclicity. Recent observations by Lincoln and Forbes (1984) on pinealectomized Soay rams living outdoors suggest that although annual reproductive cycles persist, they become attenuated in amplitude, and the timing of the peak of reproductive activity is markedly premature compared to intact controls. Subtle disruptive effects of pinealectomy and ganglionectomy on the timing of seasonal reproductive and physiological rhythms have been observed in several other species, even though the experiments were conducted on animals living outdoors and thus exposed to other enviromental influences. Examples include pony mares (Sharp et al., 1979; Grubaugh et al., 1982), goats (Buttle, 1977), and deer (Schulte et al., 1981).

These observations that pinealectomy or denervation of the pineal gland by removal of the superior cervical ganglia prevent a normal response to a change in photoperiod strongly suggest that the pineal is involved in photoperiodic time measurement, rather than directly exerting a pro- or anti-gonadal influence on the reproductive axis. No efferent neural projections have been identified from the pineal gland, thus the pineal must convert information about daylength into a humoral signal (Tamarkin et al., 1985). Although several neuroactive peptides have been identified in the pineal gland, considerable evidence points to indoleamines, and in particular melatonin (N-acetyl-5-methoxy-tryptamine) as the active neuro-hormone (Reiter, 1980). Evidence comes

from two approaches. Firstly, in photoperiodic mammalian species the administration of exogenous melatonin in an appropriate temporal paradigm can completely mimic the effects of a change in photoperiod, and thus override the external photoperiod on which an animal is being maintained. Exogenous melatonin can also be given to pinealectomized or ganglionectomized animals to simulate changes in photoperiod. Studies where exogenous melatonin has been used to alter seasonality will be reviewed later in this review, in the context of how melatonin provides temporal information to the brain.

The second line of evidence is that in all mammalian species studied to date a diurnal variation in endogenous melatonin secretion exits which closely correlates with the external photoperiod. Such variations have been measured in plasma (sheep: Rollag and Niswender, 1976; Kennaway et al., 1977; Arendt et al., 1981, 1983a; Lincoln et al., 1982; horses: Kilmer et al., 1982; primates: Brainard et al., 1981; and humans: Pelham et al., 1973; Vaughan et al., 1976; Arendt et al., 1977), in cerebrospinal fluid (sheep: Kennaway et al., 1977; Rollag et al., 1978b; Macaques: Reppert et al., 1979; humans: Arendt 1978; Vaughan et al., 1978) and in urine (rats: Adler et al., 1979; Rivest and Wurtman, 1983; Lynch et al., 1981; Jimerson et al., 1977). In rodents where it has not proved possible to routinely measure peripheral levels of melatonin, diurnal rhythms of pineal melatonin content and melatonin synthesising activity have been found, for example in the rat (Wurtman and Axelrod, 1965; Ralph et al., 1971), Syrian hamster (Panke et al., 1979; Tamarkin et al., 1979), Djungarian hamster (Yellon et al., 1982) and white-footed mice (Petterborg et al., 1981; Glass and Lynch, 1982). In many cases the period of increased melatonin levels is similar to the actual length of the night, though at least in the

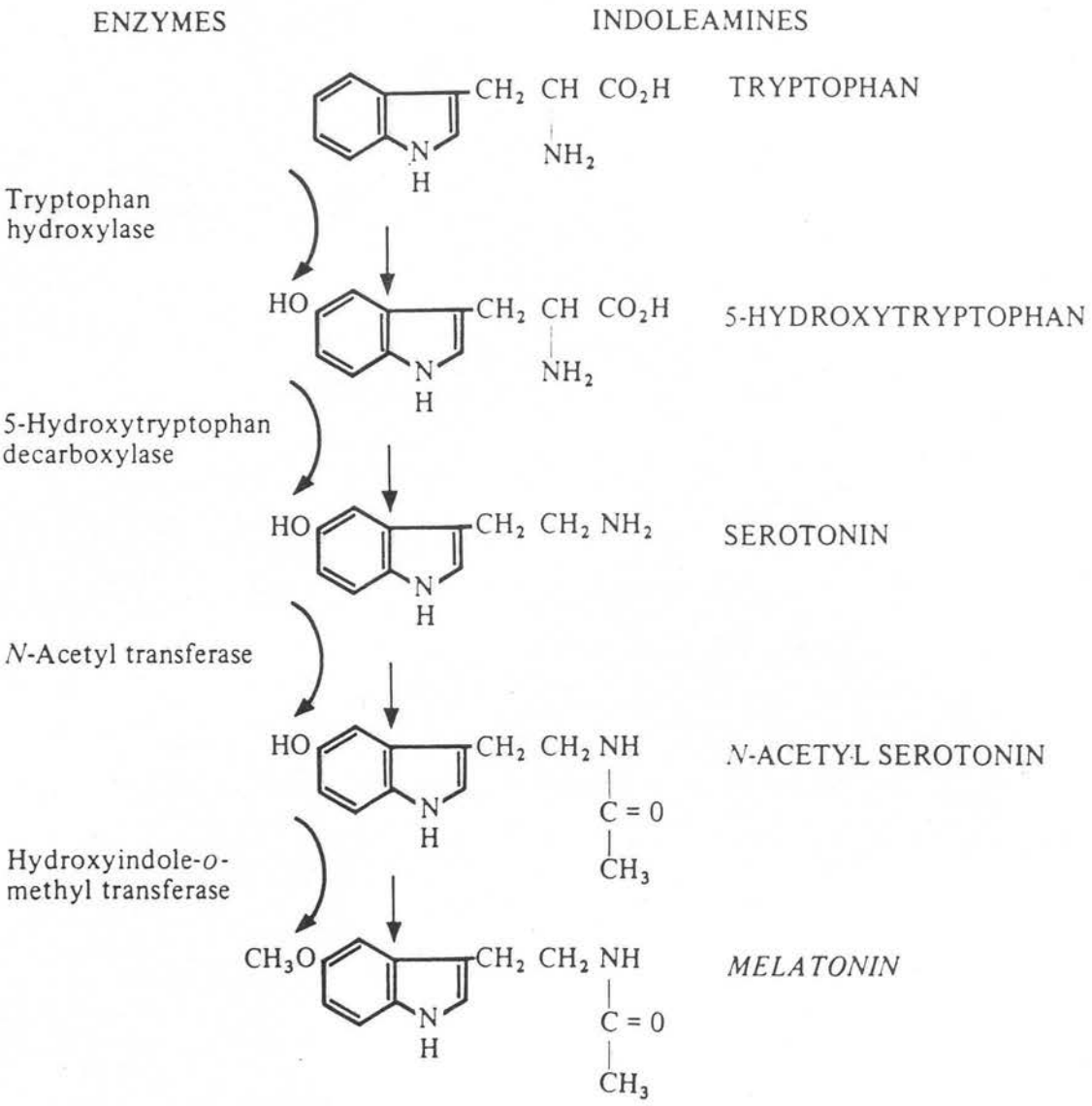

Fig. 1.1

Biosynthesis of melatonin in the pineal gland. The rate limiting enzymes are NAT (N-acetyl transferase) and HIOMT (Hydroxyindole-O-methyl transferase).

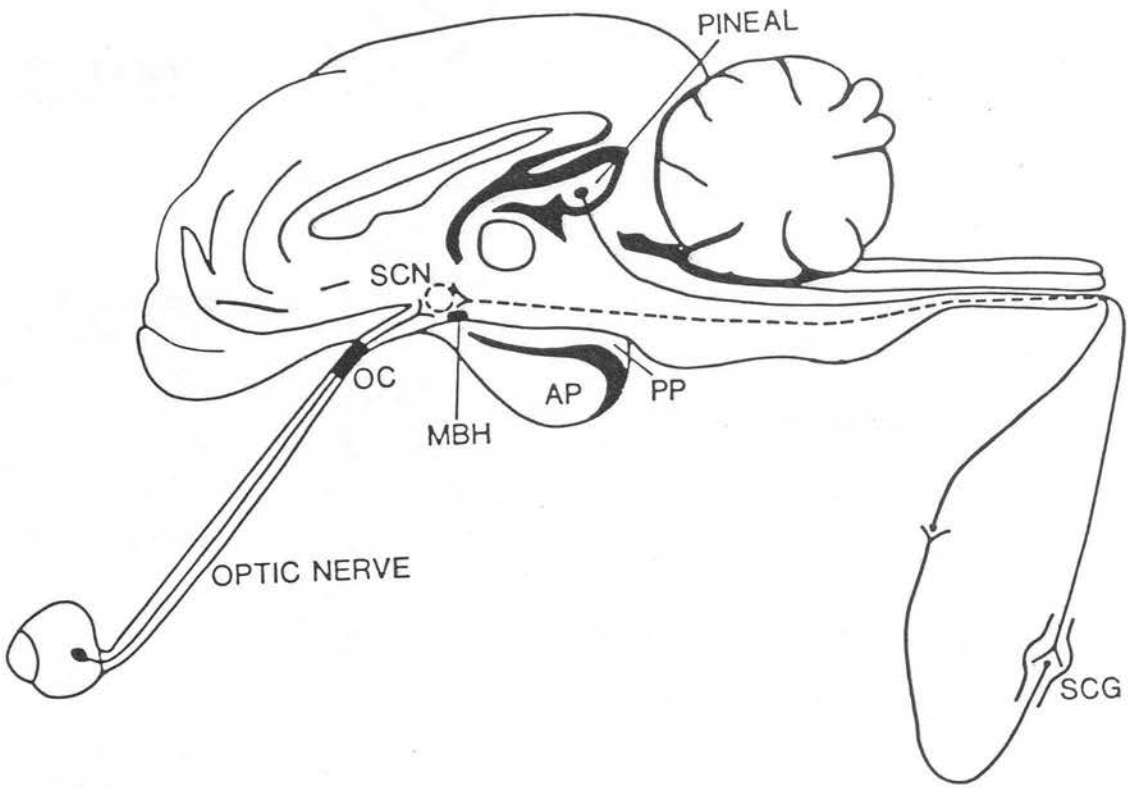

Fig. 1.2

Pathway by which photic information reaches the pineal gland. The anatomical structures linking the SCN and the sympathetic nervous system have not been identified. Reproduced by permission from Almeida (1982).

AP - anterior pituitary gland; MBH - medio-basal hypothalamus; OC - optic chiasma; PP - posterior pituitary gland; SCG - superior cervical ganglia; SCN - supra-chiasmatic nuclei.

Syrian and Siberian hamster, pineal melatonin content is only elevated for 6-8 hours, the peak being coincident with the end of the dark phase (Tamarkin et al., 1980a,b; Brainard et al., 1982b).

1.1.3 Generation of pineal melatonin rhythms.

Since the isolation of melatonin by Lerner in 1958, the biosynthetic pathway for melatonin sythesis in the pineal has been well established (see fig. 1.1). Likewise the neural pathways by which photic information from the retina reaches the pineal has been characterised (see fig. 1.2)(Preslock, 1984). What remains unclear is the mechanism by which the environmental light dark cycle controls the rhythm of melatonin secretion. Underlying this mechanism is an endogenous circadian rhythm of melatonin production. In animals transferred from regular light/dark cycles to constant dark conditions, rhythms of secretion persist, for example in sheep plasma (Rollag and Niswender, 1976; Almeida and Lincoln, 1984b), horse plasma (Kilmer et al., 1982) and in primate cerebrospinal fluid (Perlow et al., 1981). Rhythms of pineal melatonin content also persist under constant dark in rats (Ralph et al., 1971) and Syrian hamsters (Tamarkin et al., 1980a). Daily melatonin rhythms persist after blinding, though not necessarily in synchrony with the external light dark cycle (Lewy and Newsome, 1983), but not after lesion of the suprachiasmatic nuclei or removal of the superior cervical ganglia (Moore and Klein, 1974; Lincoln et al., 1982). Mammalian pineal glands cultured in vitro do not exhibit any rhythmicity of melatonin release, so it seems likely that the underlying circadian oscillator is anatomically within the suprachiasmatic nuclei (Moore and Lenn, 1972; Deguchi, 1982).

It has been suggested that light has two separate effects on the generation of melatonin rhythms; firstly a direct suppressive effect

which is intensity dependent, and secondly an indirect entraining effect whereby light sets the phase of the endogenous circadian melatonin rhythm relative to an environmental light-dark cycle (Tamarkin et al., 1985). Evidence for a direct effect of light is based on two types of observation. Firstly, several studies have reported that melatonin rhythms do not free run when animals are transferred to constant light (Rollag and Niswender, 1976; Adler et al., 1979; Tamarkin et al., 1979; Perlow et al., 1981), and secondly night interruption experiments suggest that acute exposure to light directly decreases melatonin synthesis and secretion (Wurtman et al., 1964; Klein and Weller, 1972; Rollag and Niswender, 1976; Rollag et al., 1978a; Lewy et al., 1980). These direct effects of light appear to be intensity dependent. Almeida and Lincoln (1984b) have demonstrated that melatonin rhythms in Soay rams do free run under constant light for at least ten days. The study of Perlow et al. (1981) showed that although constant light suppressed CSF melatonin levels in macaques, clear melatonin rhythms re-emerged when the animals were transferred to constant dark after three days on constant light. The implication is that constant light of a certain intensity can damp out the amplitude of melatonin secretion, but not affect the underlying endogenous rhythm generator.

In nocturnal species such as the Syrian hamster, relativly low levels (0.11-1.08 lux) of light intensity suppress melatonin synthesis (Brainard et al., 1982a), whereas in diurnal species such as the chipmunk night interruption with light does not affect pineal melatonin metabolism (Reiter et al., 1982). In humans, fluorescent light of 500 lux is unable to suppress melatonin secretion (Jimerson et al., 1977), whereas bright light of 2500 lux suppresses plasma melatonin levels to

almost day-time levels (Lewy et al., 1980). It is possible that the response to a given intensity of light pulse during the night phase depends on previous experience of "night " and "daytime" light intensities. Lynch et al. (1981) have demonstrated that the patterns of urinary melatonin excretion in rats are linked to the relative difference in light intensities rather than absolute differences. Rats housed on alternating 12 hour periods of dim light (0.2 $\mu W/cm^2$) and dark secreted melatonin during the dark period, whereas those on alternating periods of dim light and brighter light (45-110 $\mu W/cm^2$) secreted melatonin during the dim light. In three species of wild-captured squirrels it has been demonstrated that rhythms in pineal melatonin content do persist (Reiter et al., 1982, 1983). Thus rodents bred in captivity which are used to a low intensity daylight may be hypersensitive to light at night in comparison to wild rodents experiencing bright daylight. This hypothesis is supported by the observation that in laboratory raised ground squirrels (Spermophilus tridecemlineatus) pineal melatonin content is suppressed by constant illumination whereas in wild-captured ground squirrels normal pineal rhythms are observed under these conditions (Reiter et al., 1983).

Evidence is emerging that the acute effects of light on melatonin secretion also depend on the timing of the light pulse relative to the endogenous melatonin rhythm. Illnerova and Vanecek (1982) have demonstrated that a one minute light pulse early during darkness delayed the increase in pineal N-acetyltransferase (NAT) activity, however a one minute light pulse late in the dark phase produced a rapid decline in NAT activity which remained suppressed for the rest of the duration of the night. In the chipmunk, acute light pulses early in the subjective night only produce a transient, incomplete

suppression of pineal melatonin levels, whereas those given later have an immediate and profound suppressive effect (Reiter et al., 1983). The two hour bright light pulse used by Lewy et al. (1980) to suppress melatonin in humans was given 2-3 hours after subjects retired to a dark room. At the end of the light pulse plasma melatonin levels returned to normal night-time levels. In sheep too, light pulses early in the dark phase have been observed to produce only a transitory decrease in plasma melatonin (Rollag and Niswender, 1976; Rollag et al., 1978a).

The entrainment properties of light have been presumed to exist partly because of the many studies demonstrating that the onset and decline of the endogenous rhythm in melatonin secretion reflect almost exactly the onset of darkness and onset of light in their environment. Even in the rat and Syrian hamster where the peak pineal content of melatonin does not reflect the duration of the dark phase, the fall in melatonin coincides with the onset of the light period (Tamarkin et al., 1980a). Studies have attempted to demonstrate accurately photoentrainment of melatonin rhythms. Bittman et al. (1983b) transferred ewes from long days (16L:8D) to short days (8L:16D), measuring melatonin in plasma samples collected hourly for four days over this transition. This study clearly demonstrated the gradual phase advancement of the onset of the melatonin peak, some ewes establishing a coincident onset of the melatonin peak and darkness as early as day 2, and all animals doing so by day 14. Using 23 or 25 hour dark cycles which thus advanced or delayed the onset of darkness by one hour per day, Almeida and Lincoln (1985) demonstrated that Soay rams are able to correspondingly entrain their melatonin rhythms by up to an hour per cycle. Yellon et al. (1982) have demonstrated that in

Djungarian hamsters transferred from 16L:8D to constant dark thirteen hours after the onset of light (ie. darkness is advanced by 3 hours) the onset of the peak in pineal melatonin content is correspondingly phase advanced by 1.5 hours in the first cycle and the decline of the peak remains at the time of the expected dawn.

1.1.4 Interpretation of melatonin rhythms by the hypothalamus.

Two models have been proposed to explain how the pattern of pineal melatonin secretion is interpreted by the brain as a photoperiodic time cue (Lincoln, 1983). In the "duration" hypothesis it is the actual length of elevated melatonin secretion that dictates the photoperiodic response, a long duration being interpreted as a short day, whereas in the "internal coincidence" hypothesis it is assumed that there is a separate circadian rhythm of melatonin responsiveness in the melatonin target tissues, and the photoperiodic response is thus determined by the coincidence of pineal melatonin secretion with that period of responsiveness.

Most of the evidence from studies of the effect of exogenous melatonin on seasonality can be interpreted to support either hypothesis. One approach has been to use constant release implants of melatonin, thus providing an extra long (ie. 24 hour) duration of melatonin. Such implants will induce gonadal regression in both intact and pinealectomized Syrian and Djungarian hamsters housed on long days (Turek et al., 1976; Turek, 1977; Hoffmann and Kuderling, 1977) and in long day housed intact Turkish hamsters (Goldman et al., 1982). Melatorin given orally by dissolving it in drinking water has also been reported to induce gonadal atrophy in Syrian hamsters housed on long days (Pévet and Haldar-Misra, 1982). It should be noted that in certain studies in Syrian hamsters using beeswax melatonin implants,

gonadal regression was prevented in animals transferred to short days (Reiter et al., 1974, 1975). Reiter (1980) describes this effect as "functional pinealectomy" since pinealectomized Syrian hamsters also fail to regress their gonads when transferred to short days. The action of melatonin implants may depend upon dose and rate of release which may be different depending on the material used to make the implant; the study of Turek et al. (1975) indicates a complex dose-response relationship between the length of silastic melatonin implant used and the gonadal response. A satisfactory explanation for the variable effects of different melatonin implant treatments in Syrian hamsters is lacking because no data are available on the circulating melatonin levels produced by each treatment.

Many other photoperiodic species show physiological changes normally seen under short days in response to constant release melatonin implants, for example weasels (Rust and Meyer, 1969), mink (Allain et al., 1981) and the grasshopper mouse (Turek et al., 1976).

Melatonin implants induce rapid testicular recrudescence when given to rams maintained on long days (Lincoln and Ebling, 1985). Constant durations of melatonin appear to be read as short days in both "short day" and "long day" species. Suppporting evidence comes from the observations that animals cannot respond to a constant duration of melatonin if they are photorefractory at the start of treatment, thus melatonin implants given to rams previously kept on short days are unable to prevent a subsequent spontaneous testicular regression (Lincoln and Ebling, 1985), and likewise melatonin implants cannot prevent spontaneous testicular recrudescence in Syrian hamsters maintained for a prolonged period on short days. Indeed several studies have demonstrated an "induction" of testicular development by

melatonin implants in Syrian hamsters, perhaps suggesting that the extra long duration of melatonin induced a more rapid development of photorefractoriness (Turek and Losee, 1978; Turek, 1979; Bittman, 1978).

A second approach has been to give timed, discrete injections of melatonin. In long day housed hamsters a single injection of melatonin late in the light phase or early in the dark phase is very effective in inducing testicular regression whereas a similar injection late in the dark phase or early in the light phase is ineffective (Tamarkin et al., 1976). Reiter et al. (1976) observed that an intact pineal gland was necessary for this response, suggesting that the single melatonin injection in the late afternoon was coinciding with a pineal mediated rhythm of melatonin sensitivity. However, if three injections of melatonin are given to pinealectomized hamsters over the course of six hours a short day response is observed (Goldman et al., 1979). The different temporal effects of single melatonin injections could be explained by the duration hypothesis in that the exogenous melatonin injection given late in the light phase combines with the endogenous pineal melatonin secretion to produce a long duration signal whereas the early morning injection is unable to do so. A recent study, however, argues that the endogenous melatonin rhythm is necessary to set the rhythm of melatonin sensitivity. Two melatonin injections given 8.5 hours apart in the light phase to pinealectomized hamsters do not induce testicular regression, whereas if one melatonin injection is given to mimic the pineal content peak late in the dark phase, and the other is given late in the light phase (ie. the injections are still 8.5 hours apart but phase shifted relative to the light-dark cycle) gonadal regression is induced (Watson-Whitmyre and Stetson, 1983).

In intact sheep maintained on long days injection or oral administration of melatonin is highly effective in producing a short day response if given in the subjective "afternoon" (Nett and Niswender, 1982; Kennaway et al., 1982; Arendt et al., 1983b). In the latter two studies measurement of circulating melatonin concentrations demonstrated that the exogenous melatonin did indeed combine with the endogenous secretion to produce a long duration peak. In white-tailed deer oral administration of melatonin prior to the usual nocturnal rise induces a short day response (Bubenik, 1983). In the ferret, a long day breeder, injections of melatonin late in the light phase can induce premature anoestrus, also a short day response (Carter et al., 1982b.)

These studies do not necessarily reject the "internal coincidence" hypothesis. The most critical evidence has come from studies using timed infusions of melatonin. Bittman et al. (1983b) demonstrated that long duration (16 hour) infusions of melatonin given to pinealectomized ewes produced a reduction in oestradiol feedback potency, a short day response. This study did not determine whether the temporal pattern of the infusion relative to the light dark study was important, or whether a short infusion could produce a short day response. Short duration melatonin infusions were given to the pinealectomized ewes in this study late in their breeding season, but no accelerated transition into anoestrus was noted since the control animals also demonstrated spontaneous return to anoestrus associated with short day refractoriness. A follow-up study has, however, provided more convincing evidence that a short duration infusion of melatonin can produce a long day response in ewes (Bittman and Karsch, 1984; Karsch et al., 1984). Pinealectomized ewes were pretreated with a long melatonin infusion inducing a short day increase in LH levels. Those

subsequently given a short melatonin infusion showed a decrease in LH in comparison to those kept on a long infusion. A corresponding experiment demonstrated that in ewes pinealectomized on long days and given a short melatonin infusion LH levels remained low in comparison to those subsequently transferred to a long infusion. In both situations the short infusion appeared to resemble the effect of long day photoperiods which were given concurrently to control ewes. These studies do not unequivocally support the duration hypothesis because in all the experiments the long and short durations were given with a 24 hour periodicity, thus the possibility remains that the onset and decline of the induced melatonin peaks could provide stable entrainment cues as proposed in the internal coincidence model.

Data supporting the duration hypothesis has also been obtained in the Djungarian hamster, a species in which initial testicular development is under photoperiodic control (Brackmann and Hoffmann, 1977). In young hamsters raised on stimulatory long days and pinealectomized at eighteen days, infusions of 10ng melatonin over 8,10 or 12 hours resulted in testicular regression, a short day response in juveniles of this species. These effects were similar regardless of time of day of infusion, however this study did not examine whether the effects were similar if the durations were given with a non 24 hour periodicity. Infusions of 7 hours or less allowed normal testicular development to proceed (Carter and Goldman, 1981; Goldman and Darrow, 1983; Goldman et al., 1984). A long day stimulatory effect of short duration infus ions has also been demonstrated in this species. Males pinealectomized after 18 days exposure to non-stimulatory short days from birth are unable to show rapid testicular development when transferred to long days. Four or 6 hour infusions of melatonin

induced rapid testicular development, whereas 8 or 12 hour infusions resulted in continued inhibition of gonadal development (Goldman and Darrow, 1983; Carter and Goldman, 1983a,b). The duration of the peak in pineal melatonin content reflects the night length in the Djungarian hamster (Yellon et al., 1982), thus the "duration" hypothesis would appear to hold well in this species. In the Syrian hamster the duration of the peak in pineal melatonin content changes little under short and long photoperiods (Tamarkin et al., 1979; Goldman et al., 1982), however it is not possible to predict the duration of plasma melatonin rhythms on the basis of pineal content rhythms. Further elucidation of the generation and interpretation of melatonin rhythms in rodents may well await advances in techniques for observing peripheral melatonin rhythms.

If the duration hypothesis is demonstrated to be applicable to mammals in general, the interesting question arises as to the role of circadian rhythms in photoperiodic time measurement as indicated by several elegant "resonance" experiments (Elliott et al., 1972; Grocock and Clarke, 1974; Almeida and Lincoln, 1982). A possible answer is that the generation of melatonin rhythms depends on one or more underlying circadian oscillators, and that only light-dark cycles that are of 24 hours duration or multiples thereof are able to entrain melatonin rhythms to that light-dark cycle in a stable manner. The study by Almeida and Lincoln (1984a) partly supports this hypothesis; rams maintained on 8L:40D had clear circadian melatonin rhythms and thus showed rapid testicular recrudescence, whereas those on 8L:28D had disturbed melatonin rhythms, probably because the light pulse did not occur repeatedly at a similar time relative to a 24 hour circadian

clock. Under this regime the rams only showed a slow testicular recrudescence.

1.1.5 Melatonin and the release of LHRH

Studies on melatonin uptake and receptors, the effects of melatonin on neuronal electrical activity, and the effect of microinjections of melatonin into discrete brain areas provide evidence for a melatonin action within the hypothalamus, but at present do not suggest a model to explain the temporal effects of melatonin, nor do they explain the molecular basis of melatonin action (See reviews by Tamarkin et al., 1985; Glass,1984). Strong evidence indicates that in sheep it is the pulsatile release of LHRH which controls the reproductive axis via pituitary gonadotrophin release (Lincoln and Short, 1980; Goodman and Karsch, 1981; Legan and Winans, 1981), thus the action of melatonin on the reproductive axis must be to directly or indirectly affect the synthesis or release of LHRH from the hypothalamus. This review will now focus on one of the neurotransmitter mechanisms which affect LHRH release, the aim being to identify physiological systems which might subsequently be shown to be directly melatonin sensitive.

1.2 Opiates and their central actions

1.2.1 Historical background

The analgesic and physiological effects of opium, the dried extract of the poppy Papaver somniferum, were certainly known to the Greeks in 300 BC, and accounts of this substance exist in ancient Babylonian and Egyptian writings dating from 4000 BC and 2000 BC respectivly (Bowman and Rand, 1980). Laudanum, an alcoholic tincture of opium, was introduced to Britain by Thomas Sydenham in the seventeenth century, and was a popular analgesic in the nineteenth century (Crossland, 1980). A pure, alkaline substance was first extracted from opium by

Sertürner in 1803, though its structure was not established until 1925. Sertürner named the compound morphine after Morpheus, the Greek god of sleep and dreams (Way and Way, 1982). In addition to analgesia and euphoria, morphine has many other pharmacological effects within the central nervous system, including sedation and sleep, depression of the respiratory centre, depression of the cough reflex, depression of the vasomotor centre, hypotension, miosis (constriction of the pupil), and stimulation and eventual suppression of the chemoreceptor trigger zone inducing nausea and vomiting (Bowman and Rand, 1980).

1.2.2 Opiates and neuroendocrine function

Effects of morphine on the neuroendocrine function of the CNS were not described until relatively recently. Pang et al. (1977) have reviewed reports of amenorrhoea in heroin addicts dating from 1934 and 1938. In the light of previous studies where CNS depressants such as barbiturate and reserpine blocked ovulation in rats (Everett and Sawyer, 1950; Barraclough, 1955), Barraclough and Sawyer (1955) reported that morphine injected during a critical period on the afternoon of proestrus in rats would prevent subsequent ovulation, thus indicating that morphine inhibited release of "pituitary ovulatory hormone". In 1962 Meites demonstrated that daily injection of morphine into oestrogen-primed female rats would initiate lactation, indicating a stimulation of prolactin release (Meites et al., 1979). Subsequent studies in rats and man have shown that in various physiological circumstances opiates alter the release of most of the anterior pituitary hormones: acute administration generally elevates PRL, GH and ACTH, and suppresses LH and TSH (Van Vugt and Meites, 1980; McKnight and Kosterlitz, 1980).

1.2.3 The development of opiate antagonists

The major medical problem associated with the use of opiates as analgesics is that they produce tolerance and dependence (Crossland, 1980) and thus the social problem of addiction. Way(1981) suggests that the primary incentive for developing opiate antagonists was to find analgesics that were non-addictive. Pohl first reported in 1915 that an allyl substituted alkaloid (N-allyl norcodeine) would antagonise respiratory depressant effects of morphine in rats and dogs. In the 1940's Leake's group first synthesised nalorphine (N-allyl normorphine) which was found to be a potent antagonist, and is still used in the treatment of acute opiate overdose (Way, 1981). In common with most antagonists in pharmacological systems, nalorphine possesses some agonist activity. Nalorphine can thus be used as an analgesic, however this is accompanied by dysphoria and hence is unsatisfactory.

The synthesis of the "pure" antagonist naloxone (N-allylnoroxymorphine) is of major significance, not only as a treatment for opiate overdose and addiction, but because its pharmacological effects in subjects which had not received exogenous alkaloid opiates provided strong evidence for the existence of endogenous ligands for opiate receptors (Gold and Pottash, 1982).

1.2.4 The discovery of endogenous opioid peptides

The existence of endogenous opioid peptides (EOP) was predicted before their eventual discovery on the basis of two lines of evidence. Firstly bioassays had been developed where opiate activity dose-dependently inhibited electrically induced contraction in mouse vas deferens and guinea pig ileum. The pharmacokinetics of alkaloid agonists and antagonists in these systems demonstrated specific opiate receptors, so an endogenous ligand for such receptors seemed likely (McKnight and Kosterlitz, 1980). Secondly it was discovered that

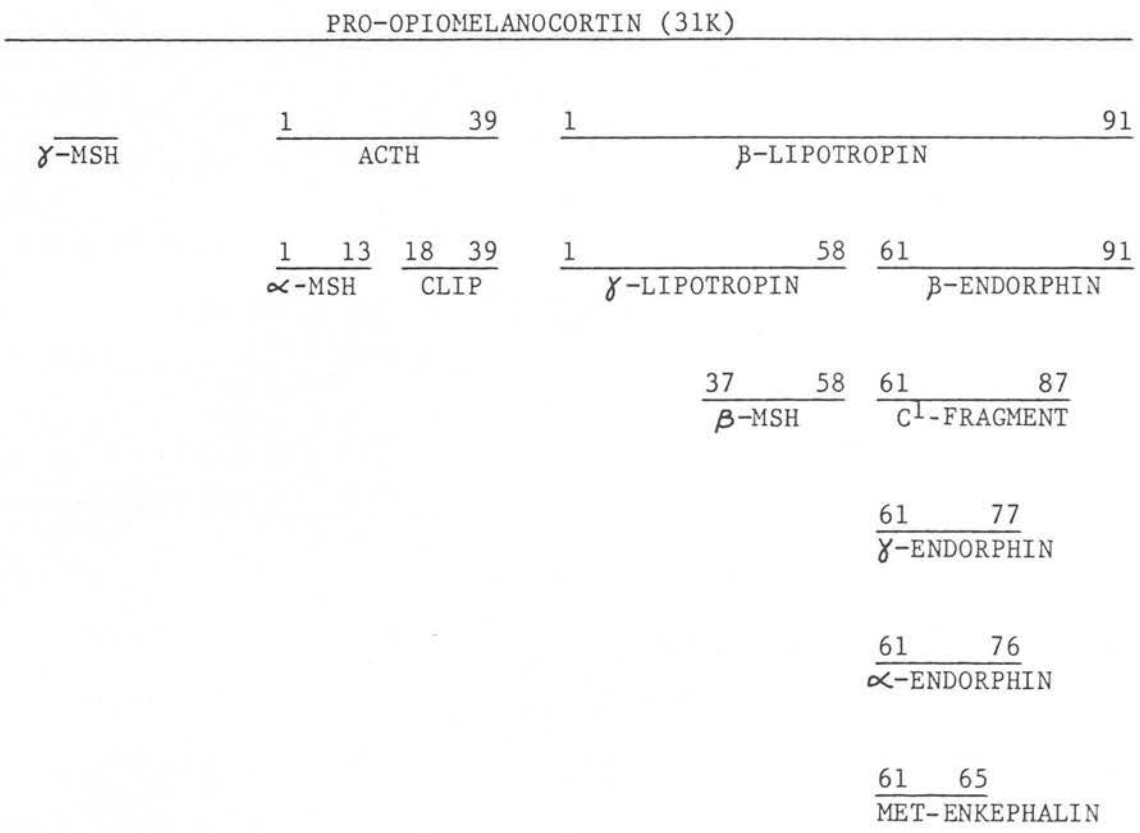

Figure 1.3

Structural relationship betwen peptides derived from pro-opiomelanocortin.

ACTH - adrenocorticotrophin; MSH - melanocyte stimulating hormone.

electrical stimulation in the periaqueductal region of the rat brain could evoke analgesia in the same way as morphine administration. This electrically evoked analgesia could be reduced by naloxone, indicating that the stimulation had released natural opiates (Akil et al., 1976; Mayer and Hayes, 1975). A third line of evidence, perhaps retrospective, is that naloxone administered alone to normal animals produces substantial endocrine responses, although few behavioral responses have been observed (Holaday and Loh, 1981)

All endogenous ligands discovered so far for opiate receptors have been peptides. Two pentapeptides, met-enkephalin and leu-enkephalin were the first to be isolated and characterised (Hughes et al., 1975). The homology of their sequences and residues 61-65 of B-lipotropin, first isolated and sequenced ten years previously (Li et al., 1965), were immediately apparent. β-endorphin (="endogenous morphine") was sequenced and synthesised the following year (Li and Chung, 1976 ; Li et al., 1976), a potent opioid peptide with an identical sequence to residues 61-91 of β-lipotropin. Three major families of EOP derived from three separate precursors have now been identified, thus although the met- and leu-enkephalin sequences resemble those in the endorphin and dynorphin peptides, their in vivo biosynthesis is likely to be entirely from the pre-proenkephalin precursor (Nakanishi et al., 1979; Höllt, 1983; Rees, 1984). Fig. 1.3 summarises the relationships of pro-opiomelanocortin derived peptides.

1.2.5 EOP inhibition of LHRH secretion

The Rat

The original observation that morphine can inhibit ovulation was confirmed by Pang et al.(1977), who further showed that this is due to the inhibition of the pre-ovulatory LH and FSH surge. In inhibited

rats, the pituitary is still fully capable of responding to exogenous LHRH, implying a central inhibition of LHRH release by morphine. This effect is naloxone reversible (Packman and Rothchild, 1976).

In adult male rats alkaloid opiates such as morphine and methadone suppress plasma LH levels and this effect can be reversed by concurrent administration of naloxone. Of major interest is the finding that naloxone alone will elevate plasma LH levels (Cicero et al., 1976; Bruni et al., 1977). It has therefore been suggested that EOP exert a tonic inhibition of LH secretion. This inhibition is within the hypothalamus since naloxone does not alter basal or LHRH stimulated LH release from rat pituitaries in vitro (Cicero et al., 1979). Two studies report that naloxone (2mg/kg b.w.) does not elevate plasma LH in prepubertal male rats (Blank et al., 1979; Ieiri et al., 1979), and likewise morphine (2mg/kg b.w.) is unable to suppress plasma LH. Female prepubertal rats were, however, responsive to naloxone. In contrast, Wilkinson and Bhanot (1982) report that in gonadectomized prepubertal male and female rats the long acting enkephalin analogue FK-33824 will inhibit LH secretion, and that increased doses are needed as the rats progress through puberty. They therefore suggest that changes in gonadal steroid feedback during puberty are due to a reduced EOP inhibitory mechanism. Several studies have investigated the relationship between EOP inhibition and gonadal steroid feedback. Cicero et al. (1979) and Van Vugt et al. (1981a,1982) report that naloxone can block the testosterone implant induced suppression of LH in castrate male rats, thus implying that steroid feedback occurs through an EOP mechanism. Cicero et al. (1980) also reported that naloxone can markedly elevate plasma LH in rats 48 hours after castration. In ovariectomized female rats naloxone will elevate

plasma LH, a consequence of increased amplitude and marginally increased frequency of LH pulses (Sylvester et al., 1982). This latter study equally demonstrated that naloxone can block the LH suppression produced by estradiol benzoate implants in ovariectomized rats. Clearly the response to opiate agonists and antagonists following gonadectomy is time dependent, thus Bhanot and Wilkinson (1983) report that in both male and female rats, two days after gonadectomy, naloxone increases and enkephalin analogues decrease plasma LH. No effects of these treatments can be seen three weeks after gonadectomy. Petraglia et al. (1984) report similar findings, and also observe that steroid priming of long term gonadectomized male and female rats restores the response to naloxone. A criticism of all the studies using opiate agonists and antagonists is that measuring plasma LH does not give a direct measurement of LHRH release. Following gonadectomy substantial changes in pituitary responsiveness may occur (Clayton and Catt, 1981), thus apparent decreased responses to naloxone in long term gonadectomized rats may be attributable to decreased pituitary response to hypothalamic LHRH. In a recent study which compared pituitary stimulation tests with naloxone tests, a loose correlation between naloxone response and pituitary response was observed, particularly in long term castrate male rats, however the authors conclude that changes in the pituitary LH pool and pituitary responsiveness are not sufficient to explain the changes in naloxone responses (Petraglia et al., 1984). A second important criticism is the interpretation of results based on a single or infrequent samples after a naloxone or agonist treatment. LH is known to be secreted in a pulsatile manner in rats (Gay and Sheth, 1972; Steiner et al., 1982;

Ellis et al., 1983) thus single sample studies provide little information on the actual mechanism of opioid inhibition. By collecting serial blood samples Kinoshita et al. (1980) demonstrated that β-endorphin injected into the lateral ventricle of rats reduce LH pulse frequency, and naloxone reversed this effect. It is doubtful whether infrequent samples could detect a stimulation of LH release in castrate rats where LH pulsatility is already high, thus the suggestion by Bhanot and Wilkinson (1984) and Petraglia et al. (1984) that naloxone cannot stimulate LH release in castrate rats is questionable. A study by Sylvester et al. (1982) indicates that naloxone does have subtle stimulatory effects on both LH pulse frequency and amplitude in long term castrate rats.

Man

Clinical trials have demonstrated a similar EOP inhibition of LH release in man. In adult men the long acting met-enkephalin analogue DAMME reduces plasma LH, and naloxone can reverse this suppression (Stubbs et al., 1978). Alkaloid opiates also suppress plasma LH in a naloxone reversible manner (Delitala et al., 1983b), and naloxone given alone significantly elevates plasma LH (Morley et al., 1980; Grossman et al., 1981; Zano boni et al., 1981), this elevation being a consequence of increased LH pulse frequency (Moult et al., 1981). DAMME does not affect the pituitary response to a LHRH challenge, suggesting that the site of opiate inhibition is within the hypothalamus (Grossman et al., 1981). One recent study has suggested that in the absence of gonadal steroids the response to naloxone is lost. 20mg of naloxone produced a significant increase in serum LH in normal young men, but had no effect in three young men who had been castrated at least six months before the study. Steroid replacement therapy (200mg testosterone enanthate once per three weeks for four

months) restored the response to naloxone (Foresta et al., 1983), thus the authors conclude that EOP may mediate steroid feedback in man.

Evidence exists in women that EOP inhibition of LH release varies throughout the menstrual cycle (Ferin, 1984). In studies of the effect of naloxone on pulstile[a] LH secretion at different stages of the menstrual cycle in normal women and chimpanzees, small responses are seen in the early follicular phase (Moult et al., 1981; Gosselin et al., 1983). In the late follicular phase a more significant increment in plasma LH is observed, and in the mid luteal phase naloxone induces very significant increases in both LH pulse frequency and amplitude (Quigley and Yen, 1980; Blankstein et al., 1981; Ropert et al., 1981; Van Vugt et al., 1984). In postmenopausal women it has been reported that naloxone cannot elevate plasma LH levels (Lightman et al., 1981b), however the naloxone response can be restored by three weeks oral treatment with conjugated oestrogens (Melis et al., 1984). In post-partum women there is also evidence for opioid inhibition of LH secretion, however this has been difficult to assess because pituitary responsivness to LHRH is extremely low, and because the LH response to naloxone cannot be accurately assessed because of cross-reacting hCG in the plasma. Nevertheless, the authors conclude that the high EOP inhibition may be a consequence of high steroid levels in pregnancy (Ishizuka et al., 1984). The observed correlation between responses to naloxone and plasma steroid levels has been taken to support the hypothesis that EOP mediate the negative feedback effects of steroids in the hypothalamus. Supporting this hypothesis are the observations in macaque monkeys that β-endorphin-like immunoreactivity in hypophyseal portal blood increases during the luteal phase of the menstrual cycle, and that in castrated macaques β-endorphin

concentrations rise following replacement of oestrogen and progesterone (Wehrenberg et al., 1982; Wardlaw et al., 1982a). Wardlaw et al. (1982b) have also observed increases in hypothalamic β-endorphin in pregnant rats. In several studies, however, correlations between steroid feedback and opioid inhibition are not so apparent. For example, patients with non-hyperprolactinaemic amenorrhoea show significant plasma LH increases following naloxone despite having lower serum oestradiol levels than those normally found in the early follicular phase (Blankstein et al., 1981). Also Lightman et al. (1981a) demonstrated that the degree of LH stimulation by naloxone in hyperprolactinaemic women was primarily related to the basal levels of LH prior to treatment, thus EOP inhibition of LH release is not obligatorily linked to peripheraloestradiol levels.

1.2.6 Site and mechanism of EOP inhibition

The Pituitary gland

Since the vast majority of studies on opioid mechanisms have measured pituitary hormone responses rather than direct hypothalamic responses the question arrives as to whether EOP might directly affect pituitary secretion. In vitro studies on the effect of opiate agonists and antagonists on PRL, GH and LHRH stimulated LH release in pituitary cultures indicate an absence of direct effects (Rivier et al., 1977; Grandison and Guidotti, 1977; Meites et al., 1979). Likewise in studies in vivo, opiates do not affect normal pituitary responses to hypothalamic releasing factors (Shenkman et al., 1972; Cicero et al., 1976; Morley et al., 1980; Grossman et al., 1981). A third, indirect line of evidence is that patients with a hypothalamic LHRH deficiency (eg. Kallmann's syndrome) are unable to respond to naloxone, even

though the pituitary contains gonadotrophins and is responsive to exogenous LHRH (Blankstein et al., 1981). Barkan et al. (1983) report that morphine can block the transitory decrease in pituitary LHRH receptors observed in ovariectomized oestradiol implanted rats prior to the LH surge. However, this study does not critically test the possibility that opiate-induced changes in hypothalamic LHRH release may have modified the number of pituitary receptors, as has been demonstrated in several studies (Clayton, 1982; Popkin and Fraser, 1983).

Hypothalamus

Several lines of evidence support the hypothesis that EOP directly modulate the release of LHRH from LHRH nerve terminals in the median eminence. Stimulatory effects of naloxone and inhibitory effects of EOP on K^+ induced LHRH release have been directly shown using in vitro perfusions of mediobasal hypothalami from rats (Drouva et al., 1981; Wilkes and Yen, 1981) and from human foetuses aged 22 weeks (Rasmussen et al., 1983). Studies on oxytocin release from posterior pituitary neurohumoral terminals also support the model of opioid neuromodulation of peptide release from nerve terminals (Clarke et al., 1979).

Indirect evidence comes from observations of neuroendocrine effects of parenterally administered β-endorphin and enkephalin analogues. It is widely reported that such endogenous opioid peptides do not penetrate the blood brain barrier (Rapoport et al., 1980; Merin et al., 1980; Houghten et al., 1980), however systemic administration of various EOP or analogues which are more resistant to peripheral degradation produce significant endocrine changes (β-endorphin: Dupont et al., 1977; Foley et al., 1979; Reid et al., 1981: met-enkephalin

analogues: Stubbs et al., 1978; Von Graffenried et al., 1978). The implication is therefore that exogenously administered EOP act on LHRH neuron terminals in the median eminence which are outside the blood brain barrier.

Considerable evidence suggests that EOP also modulate the monoamine inputs to LHRH neurone cell bodies (Kalra and Kalra, 1983). Studies where microimplants of naloxone have been put into specific hypothalamic areas of steroid-primed ovariectomized rats indicate that plasma LH is elevated by implants in the medial pre-optic area (MPOA) or the arcuate nucleus (ARC), areas known to be rich in LHRH cell bodies (Kalra, 1981). Administration of the noradrenaline (NE) synthesis blocker diethyldithiocarbamate prevents the stimulatory effect of naloxone implants (Kalra, 1981), likewise the NE synthesis blocker methyl-p-tyrosine and adrenergic receptor blockers such as phenoxybenzamine block naloxone induced LH secretion in rats (Van Vugt et al., 1981b; Kalra and Simpkins, 1981). Conversely adrenergic agonists such as clonidine increase LH secretion in morphine-suppressed rats (Kalra and Gallo, 1983).

EOP have also been demonstrated to affect several other neurotransmitter systems. Iontophoresis of morphine into the arcuate nucleus of dioestrus female rats or systemic administration of morphine reduces dopamine release into the hypophyseal portal vessels (Haskins et al., 1981; Gudelsky and Porter, 1979). In vitro met-enkephalin inhibits dopamine-induced LHRH release from rat mediobasal hypothalamus (Rotsztejn et al., 1978). The role of dopamine in controlling LHRH release is itself controversial; many in vivo studies suggest an inhibitory action of dopamine on LH secretion, especially in humans (Leblanc et al., 1976; Judd et al., 1978). Delitala et al. (1980)

report that dopamine inhibits the naloxone-induced gonadotrophin rise in man, however the same authors later report the failure of the dopamine antagonist metoclopramide to enhance the naloxone response (Delitala et al., 1983a) and conclude that opioidergic mechanisms do not function via dopaminergic neurons.

Several studies suggest that opioids may affect serotonergic mechanisms. Alkaloid opiates such as heroin and morphine increase synthesis of serotonin from its tryptophan precursor in rat brain (Perez-Crust et al., 1975), and Van Loon and De Souza (1978) have demonstrated that β-endorphin injected intracisternally will increase serotonin release and turnover in rat brain. In studies with intact prepubertal female rats, Ieiri et al. (1980) demonstrated that pretreatment of animals with the serotonin precursor 5-hydroxytryptophan would prevent the usual naloxone-induced LH rise, and conversely pretreatment with parachlorophenylalanine which reduces hypothalamic serotonin content would augment the naloxone response. Reagents altering serotonin metabolism alone do not alter basal LH levels. The authors therefore suggest that EOP increase serotonin metabolism in the hypothalamus which thus inhibits LHRH release. As with dopaminergic mechanisms, the role of serotonergic neurons in the control of gonadotrophin release remains to be fully elucidated as both inhibitory and facilitory effects of serotonin on LH release have been demonstrated under different physiological conditions (Kalra and Kalra, 1983).

1.2.7 Distribution of EOP within the brain

Immunohistochemical studies have demonstrated that the three major groups of EOP are all widely distributed in brain tissue, although they occur in distinct neuronal systems (Watson et al., 1978). A huge

literature describes the exact localisation of enkephalinergic, endorphinergic and dynorphinergic neurons in the rat. In summary, perikarya displaying enkephalin-like immunoreactivity have been identified in the medial preoptic area, suprachiasmatic nucleus, ventro-medial and anterior hypothalamic areas, arcuate nucleus and dorsal medial hypothalamus, and in many extrahypothalamic areas including amygdala and periventricular and periaqueductal areas of the upper medulla and midbrain. Up to forty separate groups of enkephalinergic cell bodies have been described at every CNS level from spinal cord to midbrain and limbic system nuclei (Kalra and Kalra, 1983). The β-endorphin/β-lipotropin perikarya are more discrete, the major cell group being in the arcuate nucleus, with fibres extending into the periventricular nucleus, medial preoptic area, septum, suprachiasmatic nucleus, and anterior hypothalamic area (Watson and Akil, 1981). Dynorphin immunoreactivity also shows a discrete distribution within the magnocellular system and arcuate nucleus with fibres extending to the medial preoptic area (Goldstein and Ghazarossian, 1980; Watson et al., 1982).

Clearly all three EOP systems are in anatomical proximity to the diffuse LHRH perikarya (Gallo, 1980; Kalra and Kalra, 1983). Immunohistochemical studies do not provide quantitative information on distribution of EOP, nor do they neccessarily identify the actual peptides present since most antisera show significant crossreactivities with several peptides. A second approach has been to extract peptides from tissue blocks and measure concentrations of EOP by radioimmunoassay, using various chromatographic techniques to characterise the immunoreactivity. Such studies have demonstrated authentic β-endorphin in human hypothalami obtained post mortem, the

highest concentrations occuring in the arcuate nucleus and mediobasal hypothalamus (Wilkes et al., 1980a). Similar quantitative distributions of β-endorphin-like immunoreactivity have been reported in monkeys (Matsukura et al., 1978) and rats (Rossier et al., 1977a). Several studies indicate that the β-endorphin immunoreactivity in rat and monkey hypothalami is predominantly composed of β-EP_{1-31}, β-EP_{1-27} and β-EP_{1-26}, and α-endorphin, whereas other brain areas such as the hippocampus and brain stem contain higher proportions of N-acetyl-β-endorphin and N-acetyl-α-endorphin, forms which have no opiate agonist activity, though may possibly be active as opioid antagonists (Zakarian and Smyth, 1979, 1982; Wiegant et al., 1983; Akil et al., 1983).

1.2.8 EOP in the pituitary gland and peripheral circulation

In man, rats and monkeys, concentrations of β-endorphin-like immunoreactivity are several thousand times higher in the pituitary gland than in the hypothalamus (Wilkes et al., 1980a; Rossier et al., 1977a; Höllt and Bergmann, 1982). β-endorphin like immunoreactivity occurs in both the anterior and neuro-intermediate lobes of the rat and porcine pituitary, however the mechanism of processing from the pro-opiomelanocortin prohormone and mechanism of release appear to differ considerably in the two lobes (Wiegant et al., 1983; Smyth and Zakarian, 1980). Immunohistochemical studies in several species have demonstrated that β-endorphin and ACTH are stored together in anterior pituitary corticotrophs (Pelletier et al., 1977; Weber et al., 1978), however radioimmunoassay of tissue extracts suggests that β-endorphin is associated with MSH in the neurointermediate lobe (Jackson and Lowry, 1980). In vitro studies suggest that release of immunoreactive β-EP from the neurointermediate lobe is under direct neural control, dopamine inhibits release, and β-adrenergic agonists stimulate release.

Release from the anterior lobe is primarily under humoral control, corticotropin releasing factor being a potent stimulator, whereas catecholamines have no effect (Vermes et al., 1980; Rivier et al., 1982). Characterization of β-EP from anterior and intermediate lobes of the pituitary suggests different processing of the pro-opiomelanocortin precursor. N-Acetyl forms of β- and α-endorphin are prevalent in the neurointermediate lobe of rat, pig and rhesus monkey pituitaries whereas β-lipotropin and β-endorphin are predominant in the anterior lobe (Smyth and Zakarian, 1980; Akil et al., 1983; Cahill et al., 1983).

Immunoreactive β-EP has been demonstrated in the peripheral plasma of man (Nakao et al., 1978; Jeffcoate et al., 1978b; Wardlaw and Frantz, 1979; Ghazarossian et al., 1980; McLoughlin et al., 1980; Wilkes et al., 1980b), other primates (Wehrenberg et al., 1982) and rats (Guillemin et al., 1977a; Höllt et al., 1978). Jeffcoate et al. (1978b) noted a strong correlation between plasma β-lipotropin and ACTH concentrations in both normal humans and those with diseases of the pituitary-adrenal axis, for example Cushing's disease and Nelson's syndrome. Treatments which stimulate the release of ACTH, such as administration of the cortisol synthesis blocker metapyrone, foot shock stress, and adrenalectomy all produce a parallel increase in immunoreactive β-EP. Likewise the synthetic glucocorticoid dexamethasone decreases plasma ACTH and β-EP together, thus many studies indicate concomitant release of ACTH and β-EP from the pituitary (Guillemin et al., 1977b; Höllt et al., 1978; Wardlaw and Frantz, 1979; Nakao et al., 1978). Since naloxone can increase plasma ACTH, and alkaloid and peptide opiates can suppress plasma ACTH by hypothalamic mechanisms (Morley et al., 1980; Gaillard et al., 1981;

Grossman and Besser, 1982) it has been suggested that pituitary opiates may relay a short loop feedback on the control of the pituitary adrenal axis, however the low penetrance of EOP across the blood brain barrier renders this improbable (Rapoport et al., 1980; Houghten et al., 1980).

A relationship between pituitary EOP and control of the reproductive axis is in equal doubt. Petraglia et al. (1982) report that gonadectomy in male and female rats significantly lowers both pituitary and plasma concentrations of immunoreactive β-EP by three weeks post-castration and that subsequent steroid therapy will elevate the suppressed levels in castrates. Lee et al. (1981) also observed a reduction in pituitary immunoreative β-EP in male rats following castration, but did not observe a similar decline in ovariectomized female rats. Decreases in plasma β-EP and B-LPH have been described following both natural and surgically induced menopause (Genazzani et al., 1981). Studies in the rhesus monkey have failed to reveal any changes in peripheral β-EP levels following ovariectomy or through the course of the menstrual cycle (Wehrenberg et al., 1982). Goland et al. (1981) also reports no change in plasma β-EP levels during pregnancy in women, though an increase was observed at delivery. Thus evidence for a direct effect of gonadal steroids on peripheral β-EP levels is equivocal, and the physiological significance of the few reported effects is unclear.

It seems unlikely that pituitary EOP affect any aspect of CNS function, however the possibility of retrograde hypophyseal portal blood flow (Bergland and Page, 1979), slight penetration of the blood brain barrier or CSF (Rapoport et al., 1980; Gerner et al., 1982) or a

site of action of pituitary EOP in the median eminence mean that such pituitary-CNS interactions cannot be entirely dismissed.

1.2.9 Aims of the present study

1) EOP

At present no studies exist on the role of EOP in modulating pulsatile LH release in a seasonally breeding mammal, thus the overall purpose of the current study is to examine the hypothesis that EOP are involved in the central control of seasonal breeding in the Soay ram. The initial aim was to administer opiate antagonists to rams at different stages of their breeding cycle to establish whether tonic inhibition of LH release by EOP existed, and if so whether the degree of inhibition varied through the breeding cycle. The second aim was to develop a radioimmunoassay for ovine β-endorphin, a peptide shown previously to be one of the major EOP in rats and man; to investigate its distribution within the hypothalamus, and to measure peripheral concentrations of β-endorphin in relation to various seasonal somatic and reproductive parameters.

2) Melatonin

At present neither the mechanism by which the enviromental light dark cycle is converted into a melatonin signal, nor the mechanism by which the hypothalamus interprets that signal is fully understood. The first aim of the current experiments was therefore to investigate the effects of skeleton photoperiods on plasma melatonin rhythms in an attempt to resolve the direct and entrainment functions of light previously described. The second aim was to investigate the effects of constant light or dark photoperiods on circadian rhythms of melatonin and reproductive cycles in an attempt to clarify the role of melatonin in photostimulation and photorefractoriness.

Chapter 2

General materials and methods

2.1 Establishment and validation of a radioimmunoassay for ovine β-endorphin

The assay described in this thesis is a modification of assays set up in the Dept. of Chemical Endocrinology, St. Bartholomew's Hospital, London, for the measurement of human endorphin-like peptides in biological fluids (Jeffcoate et al., 1978a,b; McLoughlin et al., 1980; Smith et al., 1981).

2.1.1 Buffers

The standard buffer was 0.05 M phosphate buffer, pH 7.6, containing 0.25% (w/v) bovine serum albumin (RIA grade, Sigma) and 0.025% (w/v) sodium azide (BDH, Poole). The actual assay buffer also contained 0.1% (v/v) 2-mercaptoethanol (Sigma) and phenol red (Flow Labs.) as a coloured pH indicator.

2.1.2 Standards

Synthetic ovine β-endorphin ($\beta_oEP_{1-31} = \beta_oLPH_{61-91}$) was kindly supplied by Dr. N. Ling (UCLA, USA). Solid peptide was weighed out in a polystyrene assay tube (LP3, Sardstedt) and dissolved in 0.01N HCl to give a 0.5µg/ul stock solution. 10µl (=5ug) aliquots were pipetted into 1.5ml stoppered plastic vials (LW2075 Microcentrifuge tubes, Alpha Labs), flash frozen and stored at -40°C for use as iodination aliquots. For assay standards a 10ul aliquot was further diluted with standard buffer to give a 50ng/ml solution. 1ml aliquots were flash frozen in stoppered plastic tubes and stored at -40°C for up to 12 months.

2.1.3 Iodination of β-endorphin

The chloramine T method of iodination was used. A working solution of 10mg in 10ml 0.05M PO_4 buffer was prepared just prior to use. To the iodination aliquot of $\beta_o$endorphin (5µg) was added:

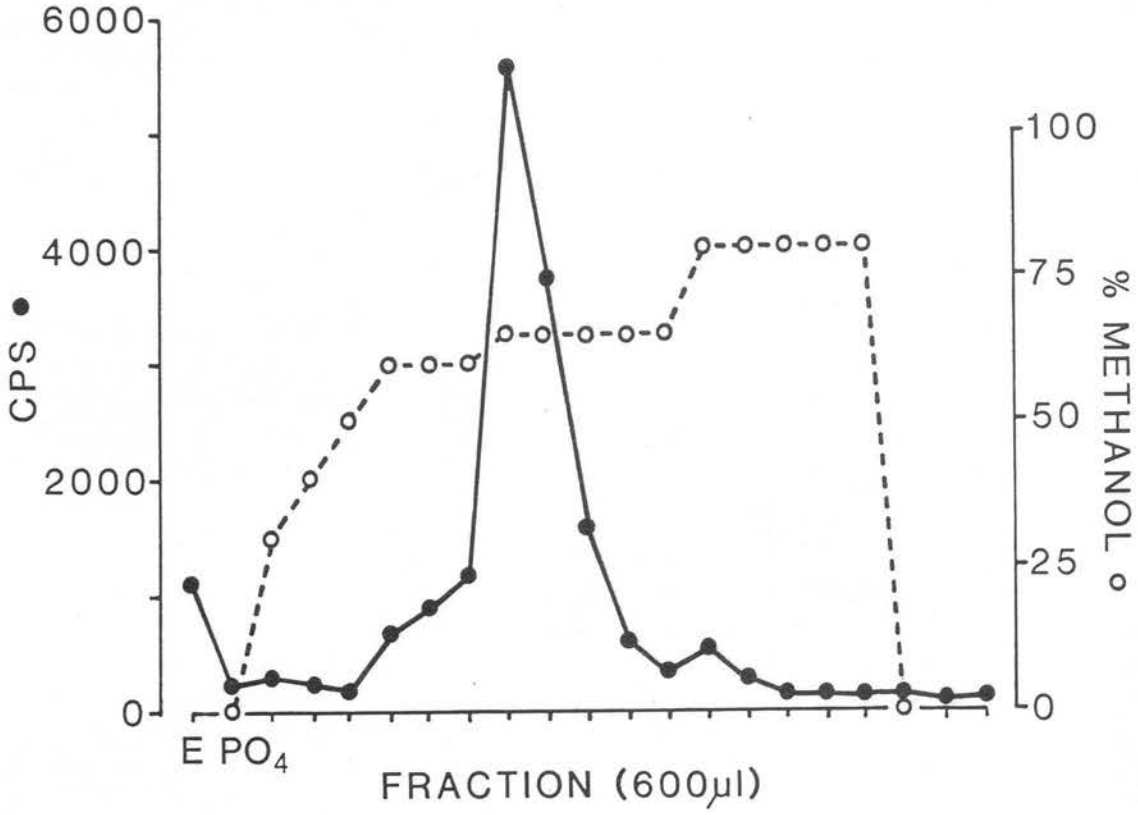

Fig. 2.1

Elution profile of ^{125}I-β_oEP tracer from an ODS column. "E" represents the initial eluate from loading the column with the iodination reaction products, and "PO_4" the initial wash with phosphate buffer. The column was subsequently eluted with an increasing gradient of methanol:water.

10µl 0.25M phosphate buffer (no BSA or preservative)

10µl $Na^{125}I$ (specific activity - 15mCi/µg iodine, Amersham)

10µl working solution of chloramine T (= 10µg)

The reaction was allowed to proceed for 30 seconds, then the solution was diluted with 500µl assay buffer and immediately loaded onto a prepared ODS column.

2.1.4 ODS column

Octodecasilicilic acid (ODS) columns were prepared by packing 10µm ODS (Phase sep.) between two filters in a disposable plastic 1ml syringe (Plastipak). The column was loaded by removing the plunger and pipetting the required solution into the syringe barrel above the filter, then replacing and gently squeezing the plunger. A set volume of 600ul was always loaded into the column. The eluate was collected in plastic LP3 assay tubes. Prior to use the column was washed with 1% (v/v) trifluoroacetic acid (TFA) (Sigma), then precoated with 10mg polypep (P5115, Sigma) dissolved in 600µl 1% TFA, followed by three washes with 80% methanol/water with 1% TFA added, and finally three washes with 1% TFA only. The precoating and washing procedure was then repeated leaving the column ready for use. The same column was used several times in succession.

2.1.5 Separation of ^{125}I-$\beta_o$endorphin from free iodine and other reaction products

The reaction mixture was loaded onto the column, then eluted with a stepped increasing gradient of methanol. The initial wash was with 0.25M PO_4 buffer, then methanol : water at concentrations of 30%, 40%, 50%, two x 60%, and several washes with 65% until the bulk of the radioactivity was eluted (see figure 2.1). The column was finally washed with 80% methanol water. All washes contained 1% TFA. The appropriate fraction(s) were made up to 5ml with 65% methanol water and

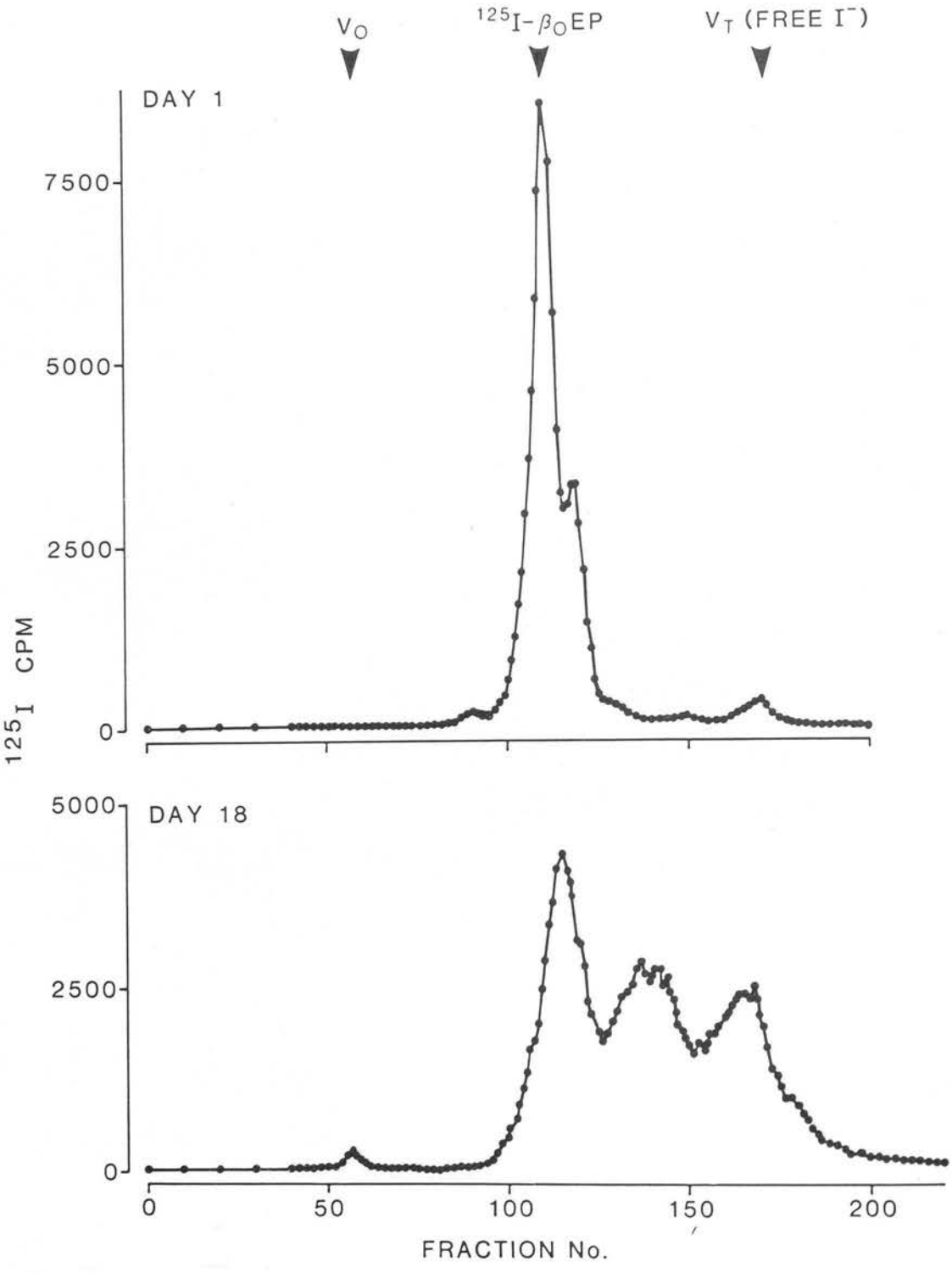

Fig. 2.2

Elution profiles of ^{125}I-β_OEP tracer run on an analytical Sephadex G-50 superfine column following purification on an ODS column. The upper panel shows tracer which was run 1 day after preparation, and the lower panel shows tracer which had been stored at -20°C for 18 days.

up to 10ml with assay buffer, and were stored liquid at -20°C. The bench life was found to be 3-4 weeks, but as figure 2.2 shows, even by day 17 some breakdown of the tracer had occured.

2.1.6 Specific activity

An approximate specific activity of the tracer has been calculated by making certain assumption: a) all 5µg of the iodination aliquot is in the column fraction(s) counted. This gives a conservative estimate; b) the specific activity of the $Na^{125}I$ is 15mCi/µg.

Calibration of gamma counter : 1mCi = 1.8×10^4 cp 10 seconds.

Pooled tracer fractions : 9.8×10^3 cp 10 seconds.

5 µg β_oEP reacts with $9.8 \times 10^3 / 1.8 \times 10^4$ mCi

specific activity = $9.8 \times 10^3 / 1.8 \times 10^4 \times 5 = 0.109$ mCi/µg

= 109 pCi/pg

molecular weight β_oEP = 3439

specific activity = 109/3439 pCi/pmol

= 0.0317 pCi/pmol

= 31.7 pCi/fmol

The molar ratios of peptide to iodine can also be estimated:

Total iodine used = 9.8×10^3mCi/1.8×10^4 mCi x 1/15 µg

= $9.8 \times 10^3 / 1.8 \times 10^4 \times 1/15 \times 10^{-6}/148$ moles

= 2.45×10^{-10} moles

Total endorphin used = 5µg = $10^{-6}/3434$ moles

$10^{-6}/3434$ moles react with 2.45×10^{-10} moles

1 mole βoEP reacts with 0.84 moles iodine.

This suggests that each endorphin molecule is mono-iodinated at one residue (TYR, position 1). This compares with human β-endorphin which

contains a second tyrosine residue at position 27 available for iodination (Chang et al., 1979 see chapter 4, figure 4.6.16).

2.1.7 Antisera

The original studies were carried out using the antiserum B4.2. This was raised in rabbits against unconjugated porcine β-endorphin (Smith et al.,1981), and kindly donated by Professor Lesley Rees, St Bartholomews Hospital, London. Later studies used antisera raised in Edinburgh in rams against the synthetic ovine β-endorphin conjugated to porcine thyroglobulin.

2.1.8 Preparation of conjugate

For the initial immunisation 4mg of the synthetic ovine β-endorphin (N. Ling) and 20mg porcine thyroglobulin (typeII) (Sigma) were weighed into a plastic 5ml vial (Sardstedt). These solids were dissolved in 2ml distilled H_2O containing 0.9% (w/v) NaCl. 100mg carbodimide (Sigma) was rapidly dissolved in 0.5ml dist. H_2O/NaCl by vortexing, then added to the β-endorphin thyroglobulin mixture. The mixture was vortexed and incubated overnight at room temperature. The reaction products were then loaded into pre-soaked dialysis tubing and suspended in 1 litre 0.9% NaCl for twenty four hours at 4°C, the NaCl solution being renewed after eight hours.

An emulsion was then prepared with Freund's complete adjuvant (Difco Labs, Detroit), 12ml adjuvant to 8ml aqueous conjugate. Four intact Soay rams were injected with the emulsion at several sites in the inguinal region, thus the total dose was approximately 1mg conjugated β-endorphin per animal. Serum was collected after eight weeks to determine antibody titres. A secondary immunization was carried out after twelve weeks. The method was identical to that for the first immunization except that 2mg of β-endorphin was used, and

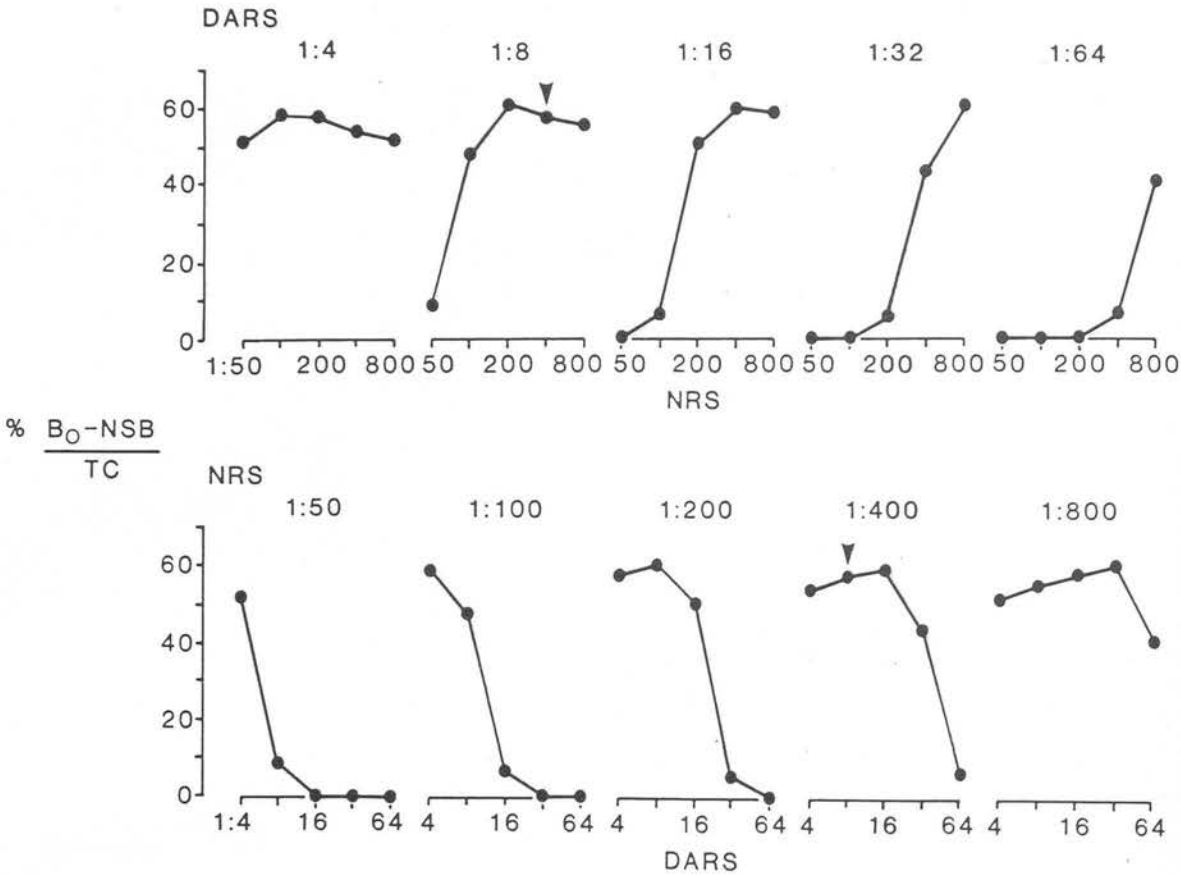

Fig. 2.3

Titration curves of second antibody (DARS: Donkey anti-rabbit serum) and carrier (NRS: normal Rabbit serum) for the immunoprecipitation of bound ^{125}I-β_OEP tracer. Optimum concentrations subsequently used are indicated by ▼.

correspondingly only half the quantities of the other reagents, and the emulsion was prepared with Freund's incomplete adjuvant (Difco Labs). Each animal was thus boosted with approximately 0.5mg conjugated endorphin. Two weeks and three weeks later 200ml blood was collected from each animal by jugular venepuncture, and the serum harvested after overnight clotting. Serum was either rapidly frozen and stored at -20°C, or diluted 1:100 with standard buffer, rapidly frozen, and stored as 1ml aliquots at -40°C.

2.1.9 Assay volumes and incubation times

The standard assay procedure adopted was to either pipette duplicate 200ul aliquots of extracted sample into plastic LP3 tubes, or where the potency of the sample was expected to fall above the range of the standard curve, a serial dilution of the sample in assay buffer was prepared. Standard curves were made by serially diluting the β_oEP standard in assay buffer from 2500pg/tube to 2.44pg/tube. Antiserum was added at an appropriate dilution in 50ul assay buffer and the tubes covered and incubated at 4°C overnight. 10-12,000 cpm tracer was added on day two in 50ul assay buffer. The tubes were again incubated at 4°C overnight before separation of bound and free tracer.

2.1.10 Separation of bound and free tracer

Two methods were tested, a second antibody precipitation of bound tracer, and a charcoal absorption of free tracer. All reagents for the second antibody technique were supplied by the Scottish Antibody Production Unit. To determine the optimum titres of second antibody and carrier, pools of B4.2 plus tracer (for total binding = Bo) and tracer alone (for non-specific binding = NSB) were incubated overnight then pipetted into 300ul aliquots. Various titrations of donkey anti rabbit serum (DARS) and normal rabbit serum (NRS) were added in 50ul

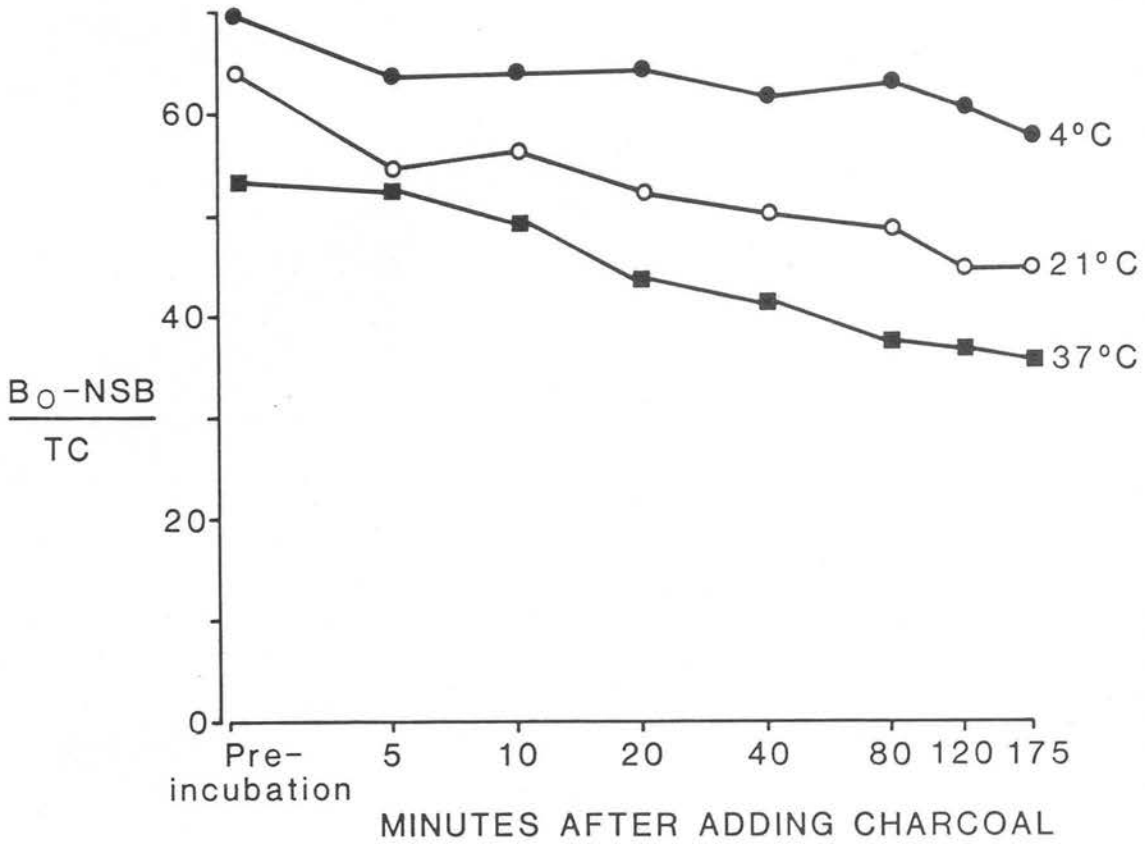

Fig. 2.4

Effect of temperature and incubation time on separation of bound and free ^{125}I-β_OEP tracer by dextran-coated charcoal. Each point is the mean of quadruplicate determinations.

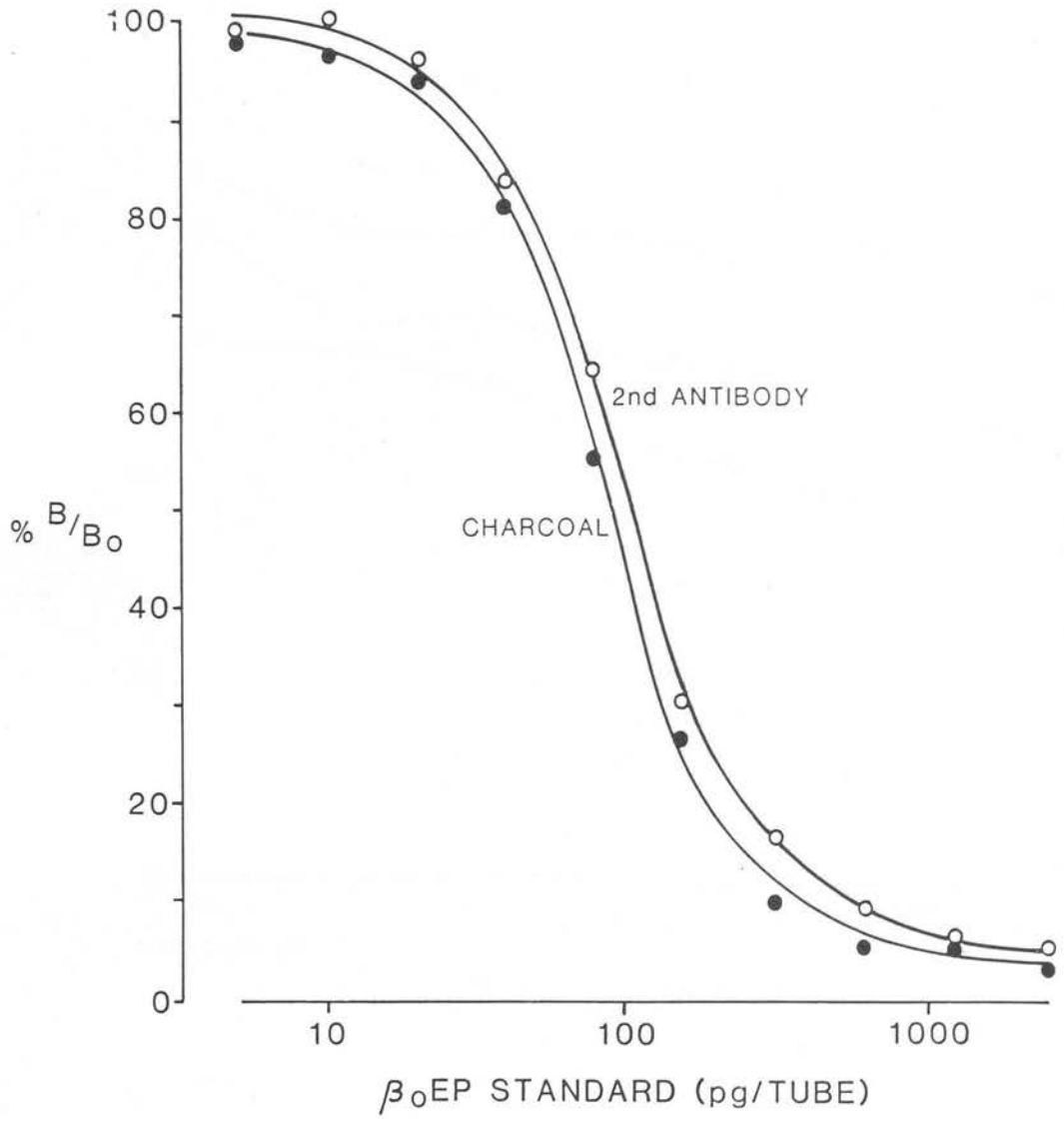

Fig. 2.5

Standard curves obtained using different methods for separation of bound and free tracer. Each point is the mean of duplicate determinations.

volumes, and the tubes incubated a further 24 hours. 1ml of 0.9% saline was added per tube. The tubes were then centrifuged at 2500 rpm at 4°C for 30 mins, the supernatants decanted, and the pellet counted. Fig 2.3 shows the specific binding (Bo-NSB) in relation to titres of DARS and NRS. Initial concentrations of 1:16 for DARS and 1:200 for NRS in 50ul were used in subsequent studies.

Titres of donkey anti sheep/goat IgG and normal sheep serum were tested in the same way to determine optimum concentrations for precipitating bound tracer in the assay system using the antisera raised in sheep.

The charcoal separation method tested was that already in use (McLoughlin et al., 1980). A charcoal suspension was prepared by first dissolving 0.75g Dextran T70 (Pharmacia) in 40ml 0.125M PO_4 buffer, then adding 10ml horse serum III (Wellcome Diagnostics) and 3g charcoal (C-5260,Sigma). The suspension was stirred for one hour in an ice bath. 200ul charcoal suspension was added per assay tube, the tubes vortexed briefly, then centrifuged at 2500 rpm at 4°C for 20 minutes. The supernatant was aspirated and the pellet counted. The possibility of charcoal stripping of bound tracer was investigated by incubating "Bo" and "NSB" pools with charcoal at three temperatures and centrifuging 300ul aliquots after various periods of incubation. Figure 2.4 shows the results. In this assay system charcoal stripping is unlikely to be a major source of variation if the charcoal and assay tubes are kept cool.

The two separation techniques were compared for sensitivity and variability. Fig 2.5 shows standard curves for a charcoal separation and for a second antibody separation for antisera B4.2 and for one of the ovine antisera. The charcoal method was adopted for all future

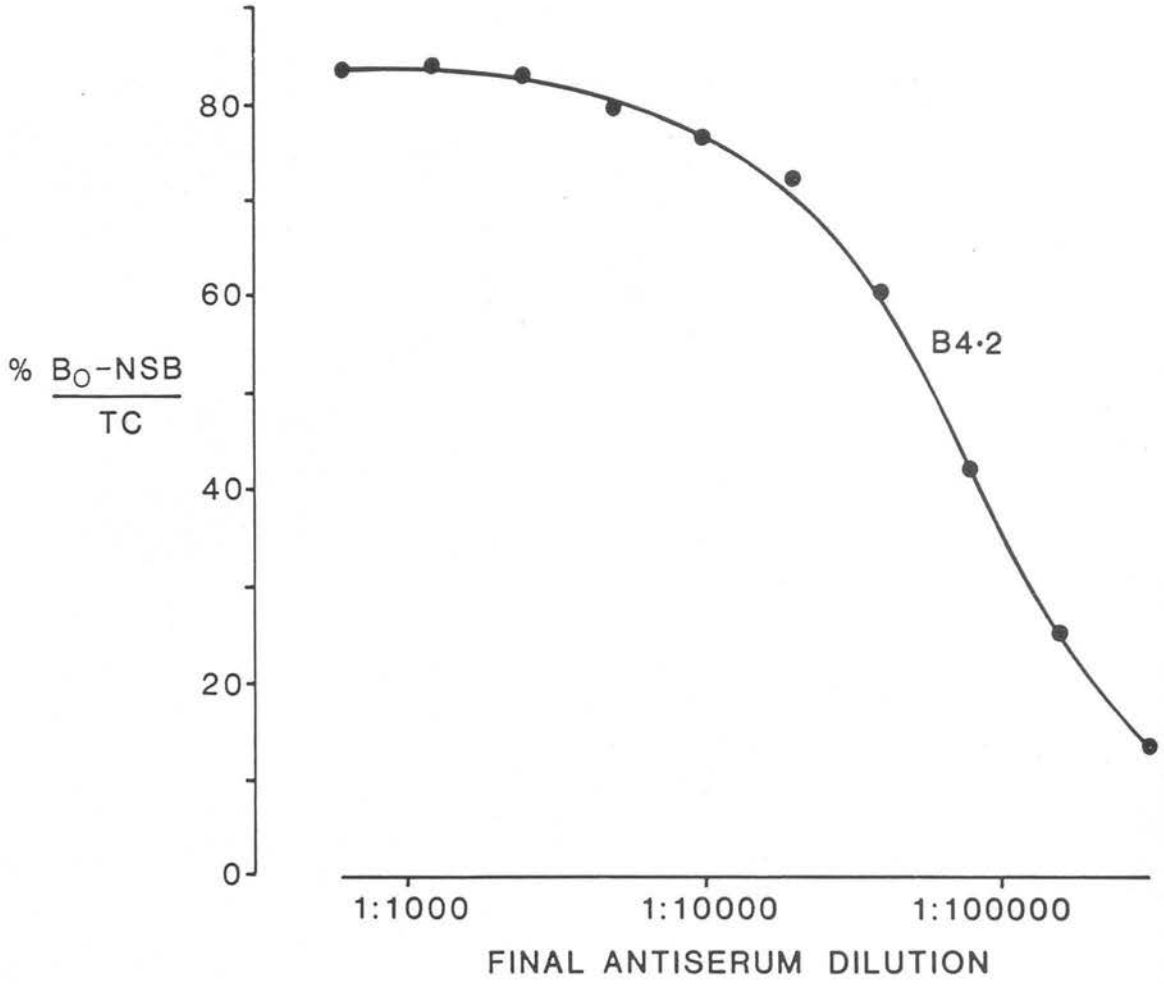

Fig. 2.7

Specific binding of ^{125}I-β_oEP by serial dilutions of antiserum B4.2. Each point is the mean of duplicate dilutions.

Table 2.8

Antiserum titres in rams immunized against conjugated ovine β-endorphin

	ANIMAL			
	2LD2	2LD9	2LD10	2LD11
21-07-83 Primary + 9 weeks	1:15000	1:17000	1:3400	1:6600
24-08-83 Secondary + 1 week	1:150000	1:160000	1:82000	1:33000
01-09-83 Secondary + 2 weeks	1:160000	1:150000	1:90000	1:60000
07-09-83 Secondary + 3 weeks	1:170000	1:150000	1:90000	1:60000

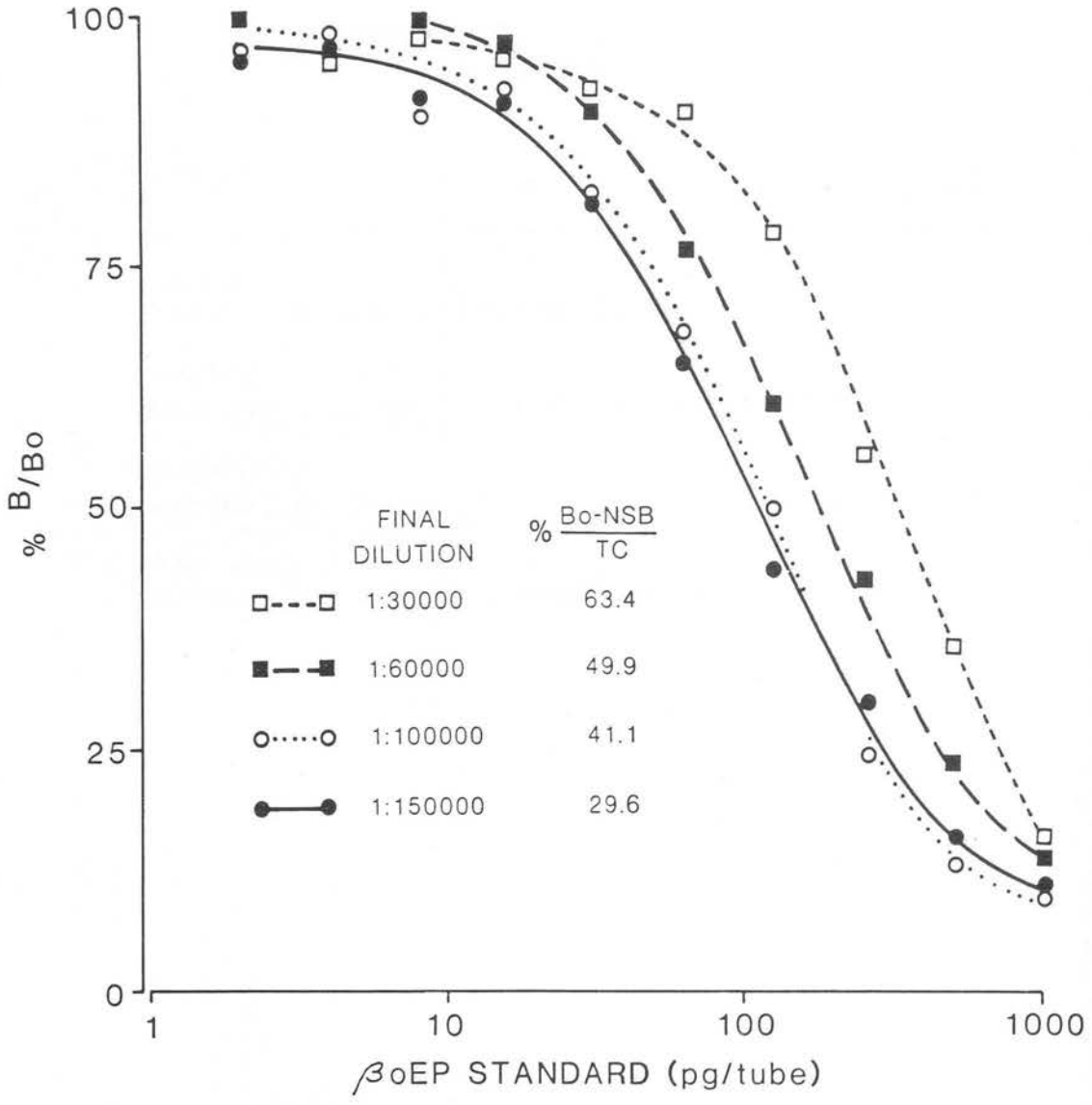

Fig. 2.9

Standard curves obtained using four different concentrations of antiserum 7.9.02. Each point is the mean of duplicate determinations.

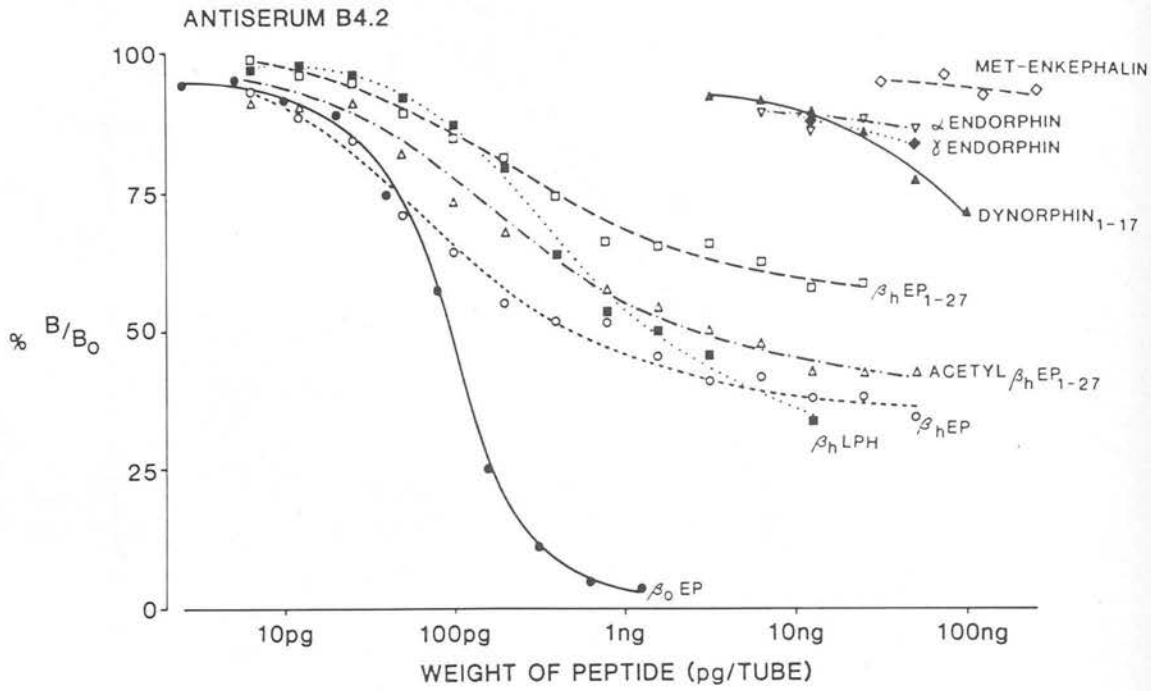

Fig. 2.10

Standard curve for β_0EP and inhibition curves for other peptides as indicated using antiserum B4.2 and ^{125}I-β_0EP tracer. Each point is the mean of duplicate determinations.

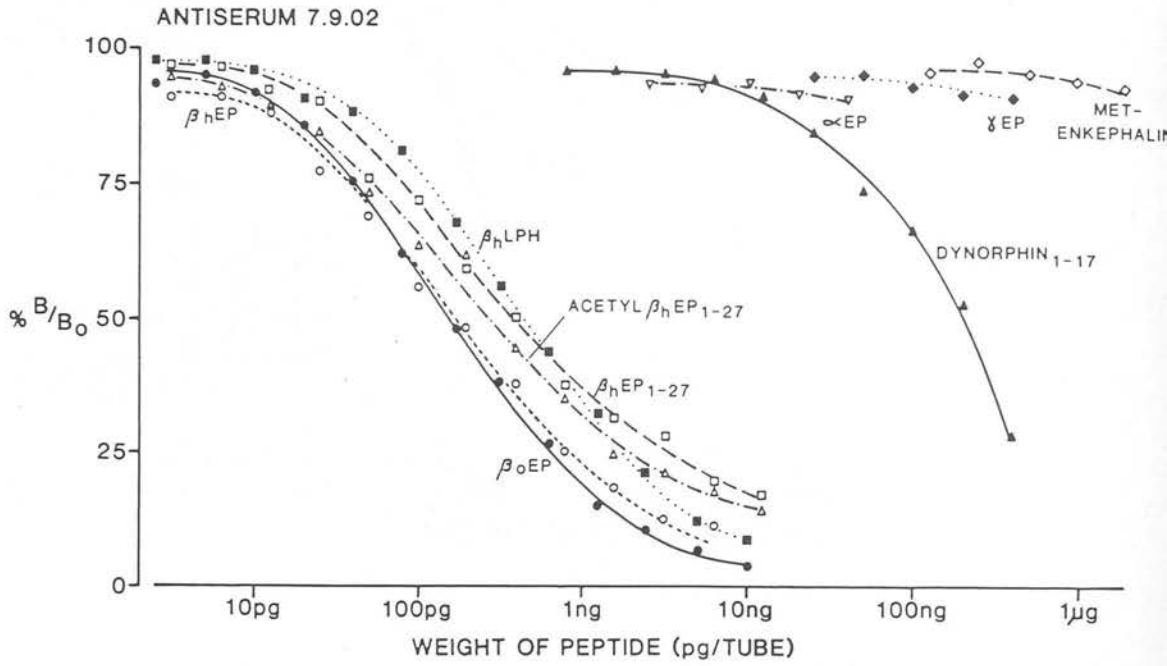

Fig. 2.11

Standard curve for β_oEP and inhibition curves for other peptides as indicated using antiserum 7.9.02 and ^{125}I-β_oEP tracer. Each point is the mean of duplicate determinations.

Table 2.12

Cross-reactivity of various peptides with antiserum raised against porcine and ovine β-endorphin.

7.9.02

PEPTIDE	% CROSS-REACTIVITY per WEIGHT	% CROSS-REACTIVITY per MOLE
Ovine β-EP_{1-31}	100	100
Human β-EP_{1-31}	100	100
Human β-LPH_{1-91}	32	100
Human β-EP_{1-27}	58	51
Human acetyl β-EP_{1-27}	42	37
αEP	<0.375	<0.19
γEP	<0.0375	<0.019
met enkephalin	<0.0075	<0.001
$dynorphin_{1-17}$	0.075	0.04

B4.2

PEPTIDE	% CROSS-REACTIVITY per WEIGHT	% CROSS-REACTIVITY per MOLE
Ovine β-EP_{1-31}	100	100
Human β-EP_{1-31}	8.75	9
Human β-LPH_{1-91}	4.04	11.8
Human β-EP_{1-27}	1.05	0.9
Human acetyl β-EP_{1-27}	1.12	1.0
αEP	<0.21	<0.12
γEP	<0.21	<0.11
met enkephalin	<0.042	<0.007
$dynorphin_{1-17}$	0.054	0.029

studies because it produced a more sensitive assay, because it was more rapid, because it gave a higher specific binding, and because the variability was comparable to that using the second antibody technique.

2.1.11 Antibody dilutions. B4.2

(See fig. 2.7). Serial dilutions were made of antiserum B4.2. These were incubated overnight with ^{125}I-β_oEP tracer, bound and free being separated by the charcoal method. For all future assays it was decided to use an initial dilution of 1:10,000 in 50ul, giving a final dilution of 1:60,000 in the total tube volume of 300ul.

2.1.12 Antibody dilutions. Ovine antisera

(See table 2.8). Antisera titres were tested on four occasions in the B-endorphin immunised rams. The titre was defined as the final dilution of antiserum required to bind 35% of the ^{125}-β_oEP tracer. Antiserum "7.9.02" was selected for further study because of its high titre. Assay sensitivity was tested at four different dilutions of this antiserum (See fig. 2.9). A concentration of 1:100,000 (final dilution) was chosen for all future studies.

2.1.13 Specificity of antisera

A variety of peptides was tested for their ability to displace binding of ^{125}I-β_oEP from antiserum B4.2 and antiserum 7.9.02 at their assay working concentration. Peptides were reconstituted in assay buffer and serially diluted in duplicate. Figures 2.10 and 2.11 illustrate crossreactivity on a weight basis. Table 2.12 gives an estimate of the molar crossreactivity of the tested compounds.

2.1.14 Calculation of assay results

After separation of bound and free tracer all assay tubes were counted in a NE 1600 gamma counter (Nuclear Enterprises, Edinburgh).

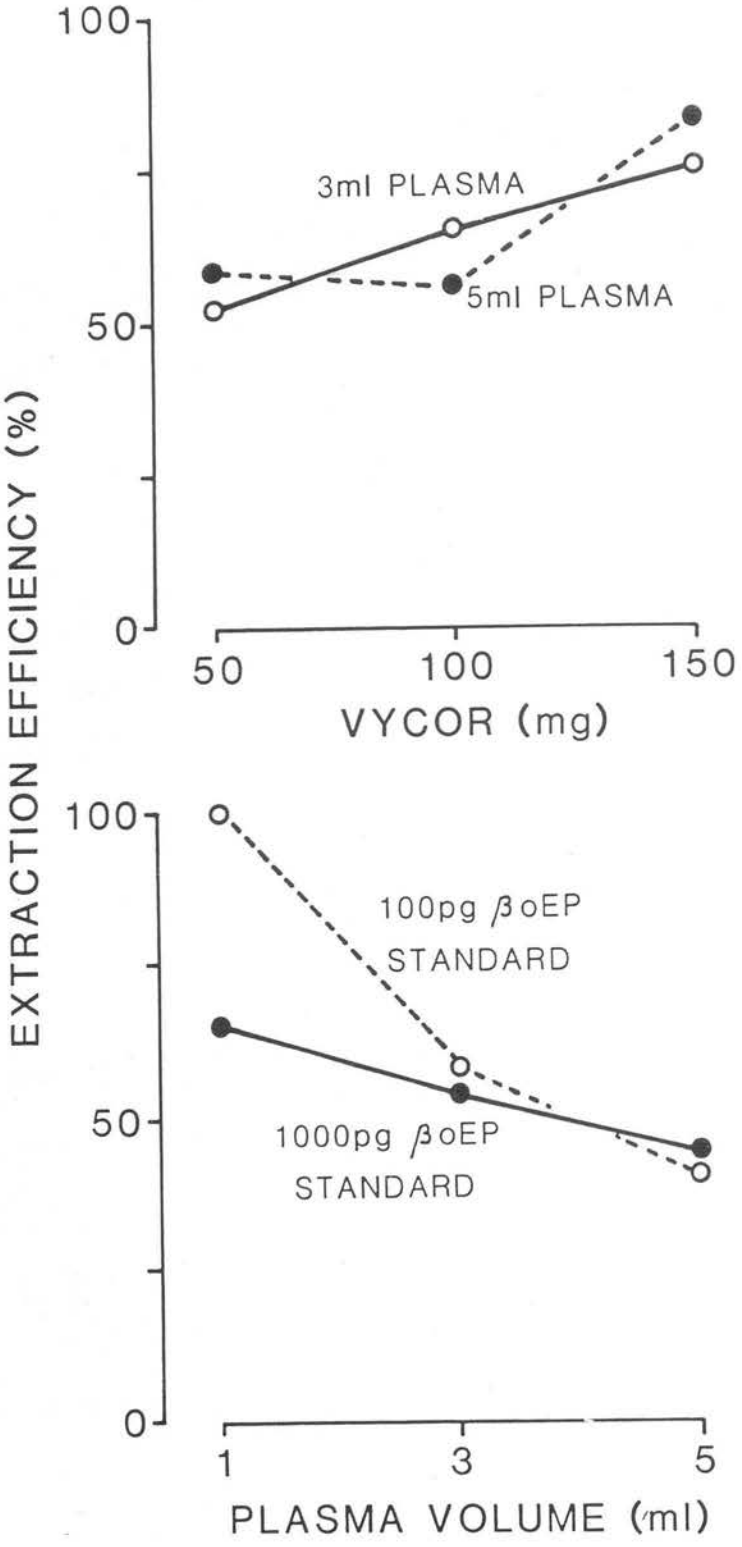

Fig. 2.13

A) Upper panel shows the effect on extraction efficiency of varying the amount of VYCOR glass used in the extraction method. Standards were extracted from either 3ml (O) or 5ml (●) plasma.

B) Lower panel shows the effect on extraction efficiency of varying the volume of plasma. A fixed amount of 100pg (O) or 1000pg (●) β_oEP standard was extracted from varying volumes of plasma.

Raw counts were processed by the RIA.GT program (Nuclear Enterprises, 1982) run on a Commodore 4320 micro-computer directly linked to the gamma counter. This program employs a log-logit transformation of the binding data for the standards and fits a regression line to the transformed data allowing calculation of unknown samples.

2.1.15 Extraction of peptides from plasma

The extraction procedure adopted was that described by Jeffcoate et al. (1978b) for peptides in human plasma. Heat activated silica glass ("Vycor", Corning Glass, New York) is added to plasma and mixed by rotation in a 10ml screw top plastic tube (Sardstedt) for 30 minutes at room temperature. After 10 minutes centrifugation at 2500 rpm the plasma is removed by aspiration and discarded. The vycor is washed by vortexing with 3ml distilled water. The tubes are centrifuged again for 10 minutes and the water aspirated. The washing process is repeated with 2.5ml 1N HCl. Finally the endorphin (and other peptides) are eluted from the vycor by adding 1ml 60% acetone:water, vortexing, then rotating for 30 minutes at room temperature. After a final ten minute centrifugation the eluate is pipetted into 75x12mm polystyrene tubes (Sardstedt) and dried down in a hot block at 60°C under a stream of oxygen-free nitrogen. The dried extract is then reconstituted in 500ul assay buffer ready for assay.

Several studies have been carried out to validate this technique for ovine plasma and to determine the optimal conditions for extraction of ovine peptides.

Figure 2.13a shows the effect of varying the amount of vycor added. 5ng of β_oEP standard was added to either 3ml or 5ml of normal sheep serum (NSS) in duplicate, and a normal extraction was carried out with 50, 100 or 150mg Vycor. Figure 2.13b shows the effect of keeping the

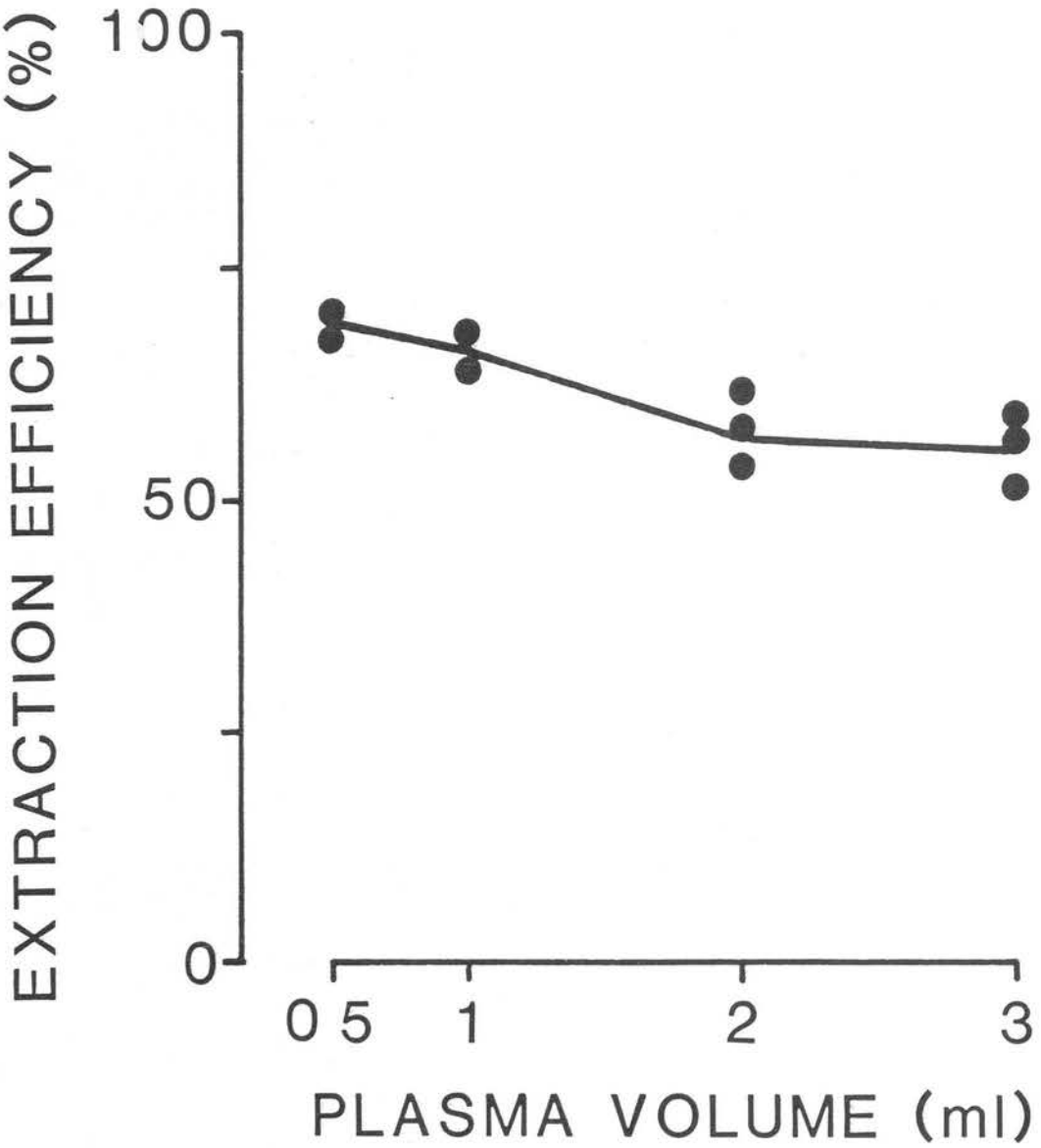

Fig. 2.14

Effect an extraction efficiency of varying the volume of plasma but keeping the total volume constant by addition of distilled water. 200pg of β_oEP standard was extracted from each tube.

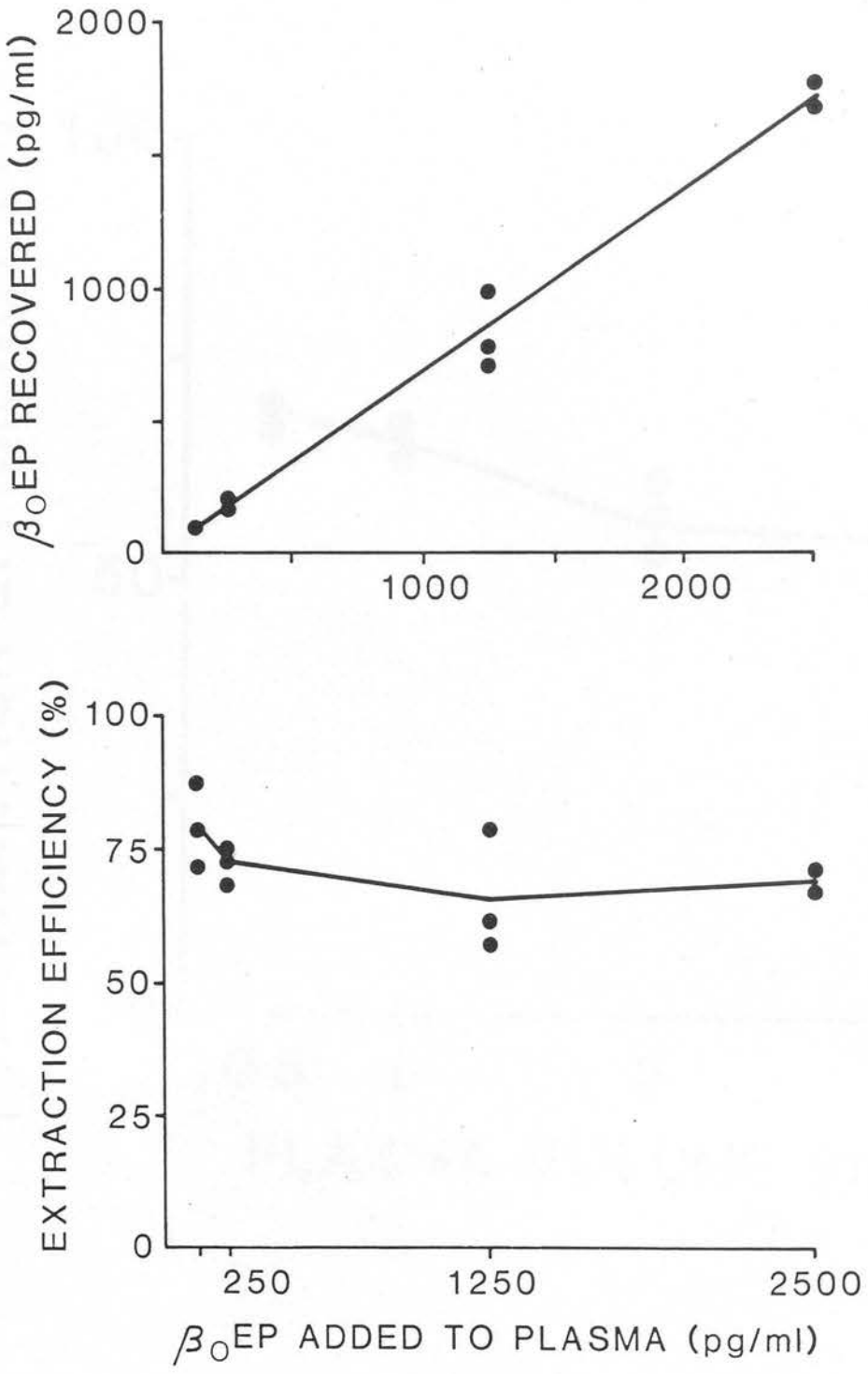

Fig. 2.15

Recovery of synthetic β_OEP standard added to a constant volume of plasma, expressed as absolute amount recovered (upper panel), and as a proportion of the initial amount added (lower panel).

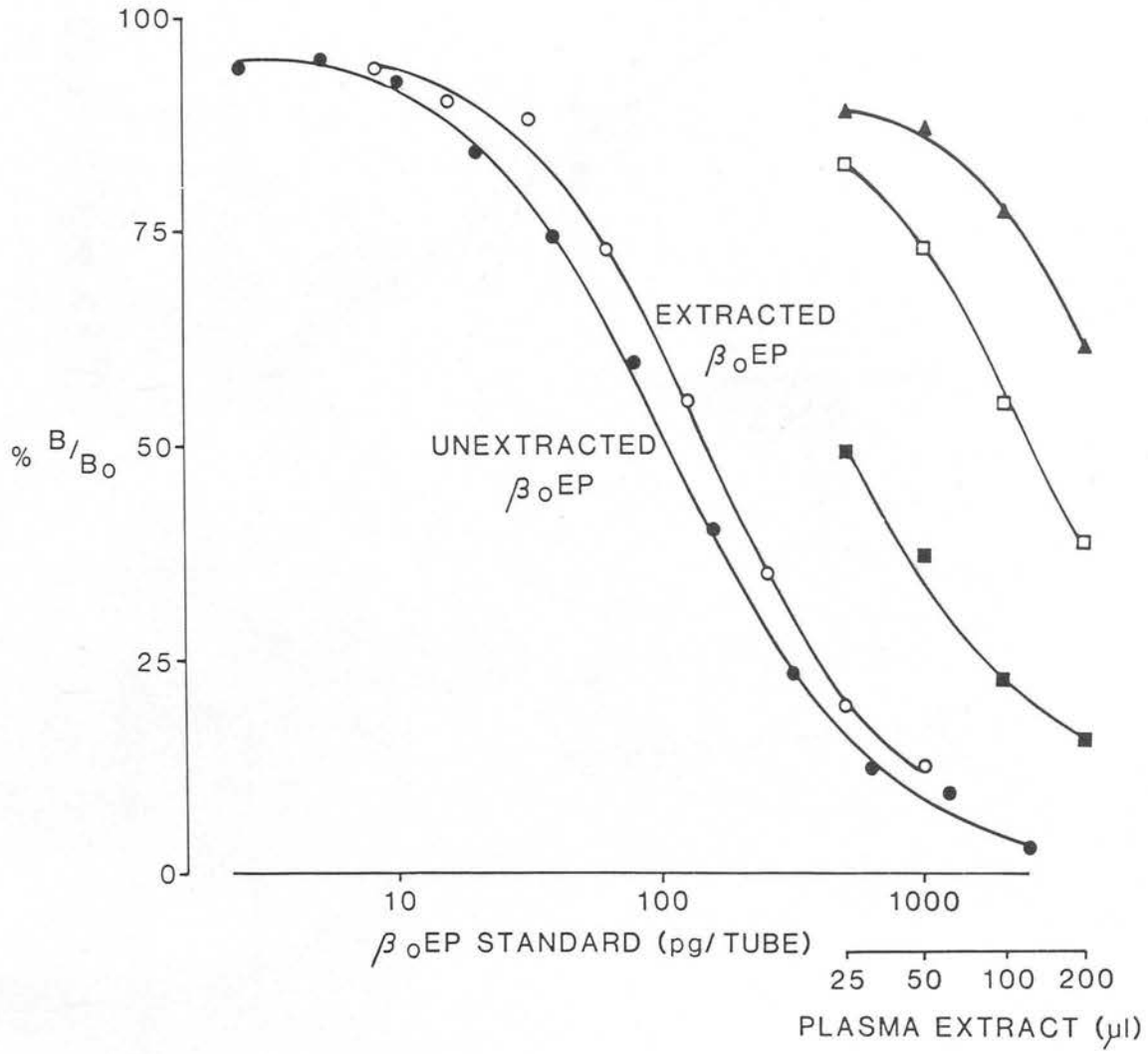

Fig. 2.16

Standard curves for unextracted (●) and extracted (○) β_oEP standards, and serial dilutions of three plasma extracts of varying potency (■ , □ , ▲). Each point is the mean of duplicate deter minations.

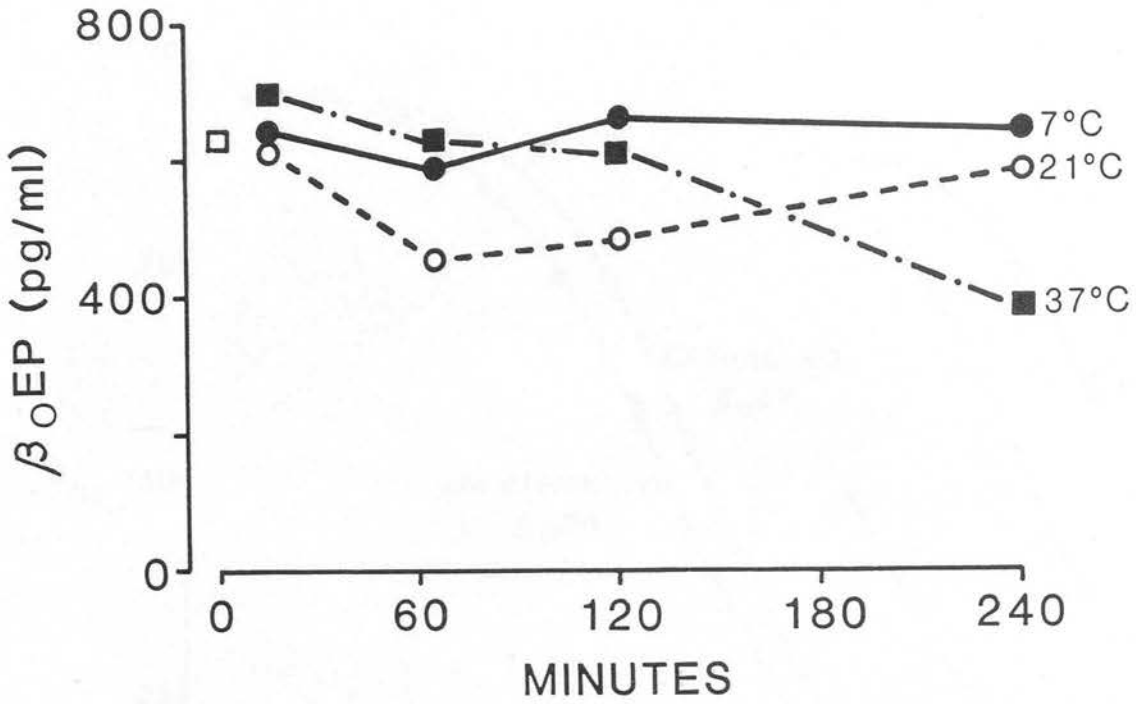

Fig. 2.17

Effect of temperature on the stability of β_OEP standard added to fresh ovine plasma. Duplicate aliquots were taken from each incubation over the course of four hours. Each point is the mean of both determinations.

weight of vycor constant but varying the volume of plasma extracted. B_oEP standard was added to normal sheep serum to create two pools of serum at 100pg/ml and 1000pg/ml. 1ml, 3ml and 5ml aliquots were extracted with 150mg Vycor, and the extraction efficiency compared. Clearly the extraction efficiency of this technique varies with the proportion of Vycor and plasma, so in all future studies fixed amounts of 3ml plasma and 150mg Vycor were used. Where less than 3ml plasma was available, the volume was made up to 3ml with water. Fig. 2.14 shows the effect of extracting different volumes of plasma spiked with 200pg/ml βoEP standard but making the volume up to 3ml with distilled water. The extraction efficiency is less variable, and in practise at least 2ml of plasma was always used in an assay extraction.

Figure 2.15 shows the extraction of different amounts of β_oEP from a fixed 3ml of plasma. There is no significant deviations from linearity over the range used. This encompasses the physiological range found in sheep plasma.

2.1.16 Parallel inhibition

Fig. 2.16 shows a typical standard curve and also the displacement of binding of the ^{125}I-β_oEP tracer by various volumes of three reconstituted plasma extracts.

2.1.17 Stability of β_oEP in plasma

Fresh plasma was collected from Soay rams and 500pg/ml β_oEP standard added. This was divided into three and incubated at different temperatures. At various time points 3ml aliquots were taken and the extraction started. Fig. 2.17 shows the results each point is a single determination. There does not appear to be any significant breakdown of β-endorphin-like immunoreactivity at room temperature.

2.1.18 Extraction efficiency and variation

Every batch of plasma extractions included duplicate internal standards comprising 5ng β_oEP added to 3ml normal sheep serum. These were used to correct for extraction efficiency within assays. Mean extraction efficiency in 25 assays was 65.1 $\pm$ 2.4%, an interassay c.o.v. of 18.0%. A low and a high quality control pool was also run in most assays, mean values being 63 $\pm$ 5.6pg/ml (n=19) and 220 $\pm$ 8.1pg/ml (n=23) giving interassay c.o.v. of 38.6% and 17.7% respectivly. To estimate a typical intra-assay variation, four low and four high quality control plasmas were assayed together, the overall mean intra-assay c.o.v. was 12.3%.

2.1.19 Collection of blood samples

Four methods of blood sampling were tested prior to actual studies. 40ml blood was collected from a jugular catheter from each of 8 Soay rams which had been cannulated the previous day, and each sample was split into four to compare the effects of collecting into plastic vs glass tubes, keeping tubes at room temperature vs on ice and flash freezing the plasma vs freezing at -20°C. After extraction and assay, data were normalised between individuals, however no significant effect of collecting protocol was observed. In all studies blood was collected straight into 10ml lithium-heparin plastic tubes (Pharmacy, Royal Infirmary, Edinburgh) kept on ice, centrifuged at 2500 rpm for ten minutes, and frozen at -20°C. In a separate study the effect of collecting into tubes containing an enzyme inhibitor was investigated. Aprotinin (A-6279, Sigma) was added to blood at 50ul/10ml blood sample, ie. approximately 60 Kallikrein Inhibitor Units per ml. This had no effect on total plasma immunoreactivity levels when compared to the untreated control samples. Aprotinin was subsequently only added to those samples collected for chromatographic characterisation.

Table 2.18

Comparison of β-EP plasma extract assays using different antisera

	Antiserum B4.2	Antiserum 7.9.02
n	13	18
% (Bo-NSB)/TC	54.8 ± 1.56	41.6 ± 1.63
50% B/Bo	66.7 ± 6.22	109.0 ± 7.06
QC LOW mean ± S.E.M.	65.9 ± 5.75	61.0 ± 4.2
Interassay C.O.V.	23.2%	20.8%
QC HIGH mean ± S.E.M.	214.6 ± 9.4	230.5 ± 11.6
Interassay C.O.V.	18.6%	11.8%
Extraction efficiency (%)	61.2 ± 2.4	67.0 ± 5.1

Bo - bound; NSB - non-specific binding; QC - quality control plasma pool; TC - total counts; c.o.v. - coefficient of variance; S.E.M. - standard error of the mean.

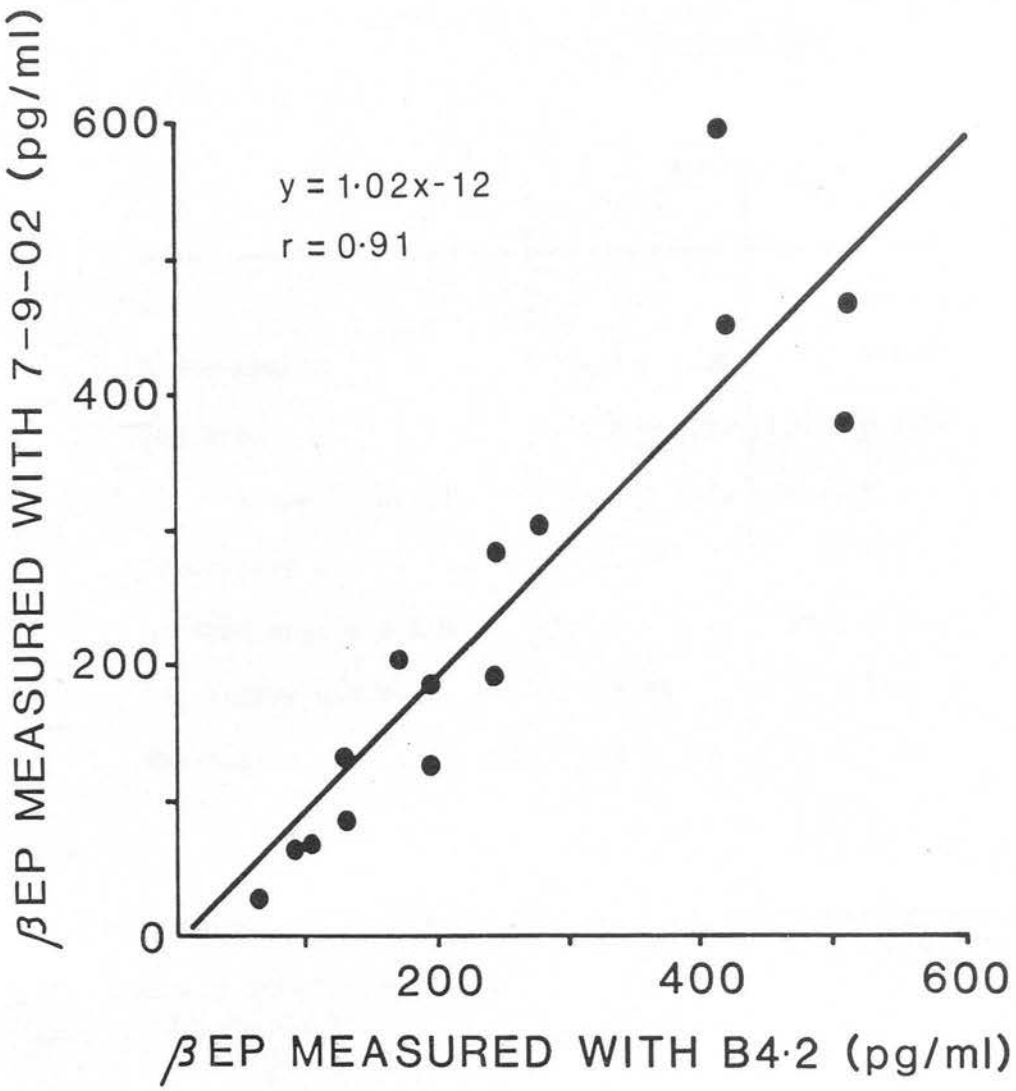

Fig. 2.19

Comparison of values for fifteen plasma extracts which were separately assayed using antiserum B4.2 and antiserum 7.9.02.

2.1.20 Storage of samples

Plasmas collected for assay were stored deep frozen at -20°C for up to 6 months. A quality control pool which was repeatedly thawed out and refrozen showed a 5-10% loss of immunoreactivity each time it was thawed and reassayed. All samples were rapidly thawed in a warm water bath prior to assay, and the β_OEP assay was always the first assay to be done on those samples collected for the measurement of several hormones.

2.1.21 Comparison of results from assays using different antisera

Table 2.18 is a comparison of various parameters for plasma extract assays using antiseum B4.2 and those using 7.9.02. To further check that the two antisera gave similar results the reconstitutes from an extraction were split in two and half assayed with each antiserum. Fig. 2.19 shows the correlation between the individual results from each assay

2.1.22 Extraction of peptides from brain tissue

Several methods for extraction of endorphin-like peptides from tissues have been described (Höllt and Bergmann, 1982; Pique et al., 1981; Zakarian and Smyth, 1979; Wiegant et al., 1983). All these methods are essentially similar in that the tissue is homogenised under acid conditions. The method adopted was a combination of previously described methods. Blocks of brain tissue are weighed and stored at -40°C in 75x12mm borosilicate glass tubes (Corning Glass, New York). 1ml 0.1N acetic acid heated to 96°C is added to the frozen tissue, and the tubes are incubated in a water bath at 94-98°C for 15 minutes. The tubes are then placed on ice, and the tissue homogenised using a sonicater (Soniprep). 30 seconds at maximum power was found to be adequate for soft brain tissue. The tubes are then centrifuged at 3000

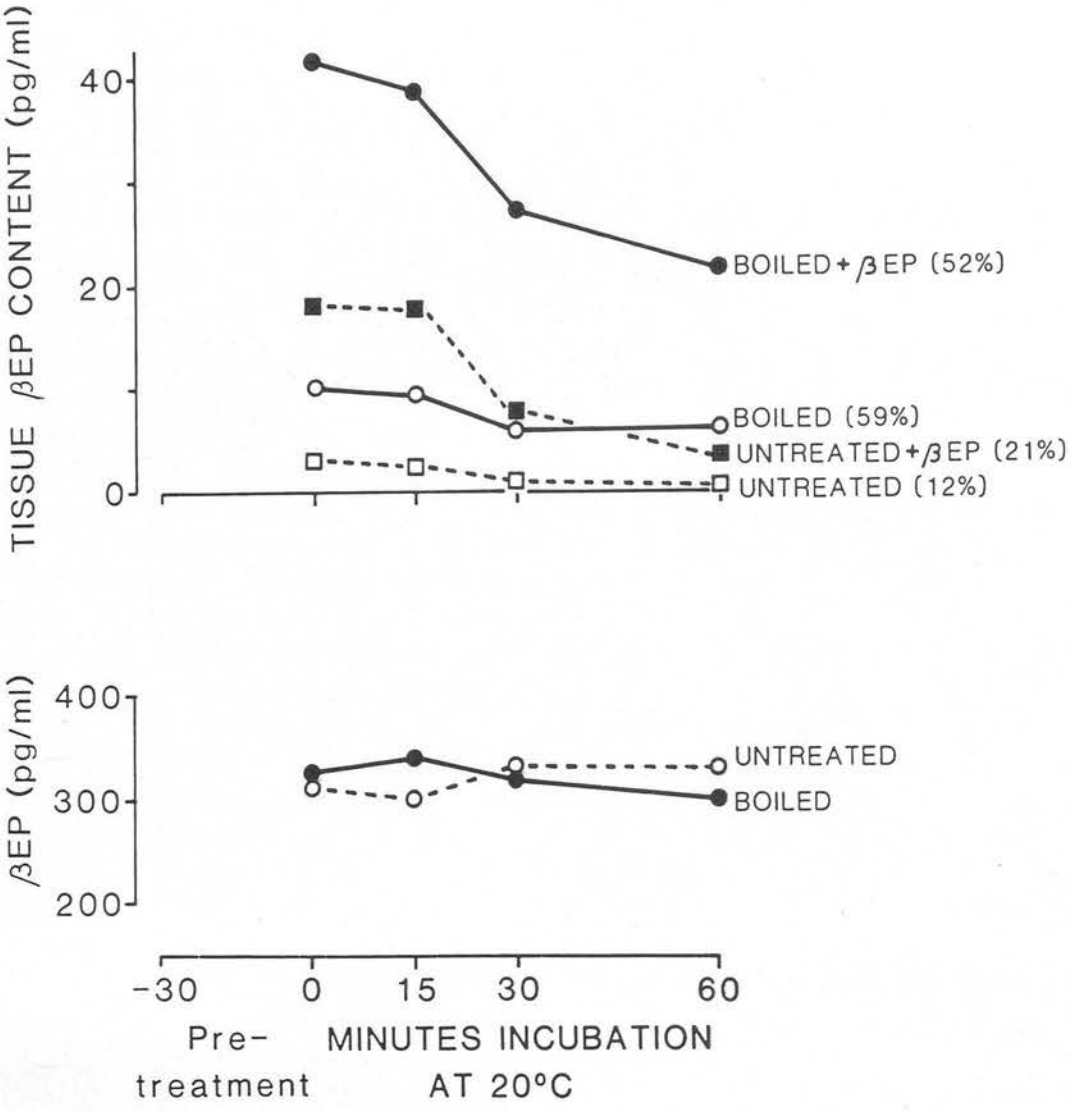

Fig. 2.20

Upper panel shows the breakdown of β-EP by hypothalamus homogenates. A pool of homogenate was prepared by sonication, and synthetic β_oEP preincubated at ≏ 100°C or 20°C for 30 minutes, and total immunoreactivity measured over the course of a subsequent hour at 20°C. Percentages indicate the amount of β-EP remaining at 60 minutes as a proportion of that at t=0.
Lower panel shows the stability of β_oEP standard added to 0.1N acetic acid which was then treated in the same way as the hypothalamic homogenates.

rpm for one hour at 4°C. 750ul of the supernatant is pipetted into a plastic 75x12mm tube (Sardstedt) and neutralised with 75ul 1N $NaHCO_3$. The neutralised extracts are centrifuged for a further 30 minutes at 3000 rpm. In duplicate, 100ul aliquots of the supernatant are diluted with 100ul assay buffer. For potent samples 50, 25 and 12.5ul aliquots are also assayed. The remainder of the supernatant is stored at -20°C for assay of other peptides or if needed for reassay.

Whereas β-endorphin-like immunoreactivity is stable in plasma, hypothalamic extracts were found to be capable of breaking down β_oEP, thus the initial boiling step is important. Fig. 2.20 shows the breakdown of β_oEP in hypothalamic homogenates at room temperature. A hypothalamic homogenate was prepared by sonicating previously frozen tissue in 0.1N acetic acid and split into two pools. β_oEP standard was added to pool A. Each pool was subsequently subdivided, half being preincubated at 96°C, the other half being stored on ice. β_oEP standard was also added to 0.1N acetic acid only and preincubated on ice or at 96°C. The preparations were then incubated at room temperature and aliquots taken, centrifuged and assayed at various time intervals thereafter. It can be seen from the percentage immunoreactivity at t=60 minutes that loss of immunoreactivity is far greater in homogenates that have not been pre-boiled. β_oEP itself is completely stable after 15 mins. boiling in 0.1N acetic acid. In a trial experiment where hypothalamic blocks were homogenised first and then subdivided to compare the effects of boiling, β_oEP immunoreactivity levels were approximately four fold higher in the blocks which had been boiled.

To estimate extraction efficiency a pool of spare non-boiled hypothalamus was homogenised and β_oEP standard was added at a

concentration of 50pg/mg tissue. In six assays the mean value was 52 ± 5.4 pg/mg, a mean extraction efficiency of 104%, interassay c.o.v. was 25.4%. The high extraction efficiency is an overestimate because the calculation of recoveries cannot take into account the evaporation of some of the original 1ml acid during the initial boiling procedure. An internal standard (5ng β_oEP in 1ml 0.1N acetic acid) was also included in each extraction. Extraction efficiency was also greater than 100% when converted back to pg/ml.

2.2 Column chromatography

2.2.1 Preparation and running of column

A glass chromatography column of 90cm x 2.2cm (internal diameter) (Wright) was packed under gravity with Sephadex G-50 superfine (Pharmacia) which had previously equilibrated in 0.05M phosphate buffer containing 0.05% sodium azide, pH 7.5 the "column buffer". The column was pre-coated with 100mg bovine serum albumin (Sigma) dissolved in 5ml column buffer.

All calibration runs and all sample runs were conducted in a cold room at a constant temperature of 4°C. The column was pumped upwards at 6ml per hour using a peristaltic pump (LKB varioperpex II). 20 minute (=2ml) fractions were collected using a LKB ultrarac. Soda glass 10ml tubes were used to collect fractions in runs using ^{125}I tracers, and plastic 5ml vials (Sardstedt) to collect column fractions for RIA in all other runs. Samples were loaded by syringe in a volume of 3ml. Between runs the column was washed with approximately 1 litre of column buffer (ie. 3 x the bed volume), and the same column was used repeatedly for 2 to 3 months.

2.2.2 Void volume

The V_o was originally determined by loading 3mg blue dextran 2000, molecular weight - 2×10^6, (Pharmacia) on the column and noting the first visible appearance in a fraction. In later runs the V_o was rechecked by loading bovine serum albumin, molecular weight 6.7×10^4 and measuring the first increase in adsorbance at 280nm using a spectrophotometer (PYE unicam SP6-500). The total volume V_t was noted by observing the free iodide ($^{125}I^-$) peak in runs using ^{125}I-β_oEP tracer.

2.2.3 Calibration

Calibration was initially carried out using two molecular weight markers, ribonuclease A, m.w. 1.37×10^4, and chymotrypsinogen m.w. 2.5×10^4 (both Pharmacia reagents). 15mg of each dissolved in 3ml column buffer was loaded onto the column, and their elution monitored by adsorbance at 280nm. The elution positions of $\beta_o$endorphin and β-lipotropin were determined by loading 2.5×10^5 cpm of the appropriate peptide tracer, and detecting gamma radiation in the column fractions. ^{125}I-β_oEP was prepared as previously described. ^{125}I-β-lipotropin was prepared from $\beta_h$lipotopin in an identical method to that for ^{125}I-β_oEP, except that separation of tracer from other reaction products was achieved by a vycor extraction as previously described for plasma. Tracer was eluted from the vycor glass with 60% acetone:water and stored as such at 4°C. For each initial calibration run tracer which had been prepared the same day was used.

Later calibration runs used native $\beta_o$endorphin and $\beta_h$lipotropin standards, their elution position being determined by RIA.

2.2.4 Preparation of plasma samples for chromatography

Blood samples for chromatography were collected with 60 KIU/ML aprotinin (Sigma). Although this had been previously shown not to alter total immunoreactivity levels in plasma, it was added in an

attempt to prevent any degradation of large precursor molecules into β-endorphin-like molecules. Plasma was extracted with vycor as previously described. Between 25ml and 60ml plasma were extracted for each column run. This was done in 5ml aliquots, and the dried down extracts were reconstituted in an appropriate volume of column buffer to ensure that the total volume of the pooled reconstituents would not exceed 3ml.

Brain and pituitary extracts were also prepared as described earlier. Since these are of much greater potency than plasma extracts, the acidic extracts were diluted to 3ml with column buffer and applied directly.

2.2.5 RIA of column fractions

Because gel chromatography results in considerable dilution of the initial sample, duplicate 200µl aliquots from each 2ml column fraction were assayed directly. Standards were correspondingly serially diluted in column buffer. The antibody was therefore added in 50µl buffer which contained five times the usual assay buffer concentration of BSA and 2-mercaptoethanol. The non-specific binding tubes also received 50µl of this buffer but with no antibody. Tracer addition on day 2 and charcoal separation on day three were as previously described.

2.3 Radioimmunoassay of Luteinizing Hormone

Blood plasma concentrations of LH were measured in a homologous ovine RIA based on that described by Scaramuzzi, Caldwell and Moor (1970). Reference standard was NIH-LH-S18 supplied by NIAMDD, Bethesda, USA. Antiserum R3 was used at a final dilution of 1:600,000. Lower limit of detection of the assay was 0.2ng/ml. Intra-assay coefficients of variation (c.o.v.) for duplicates of quality control plasma pools run in thiry-one assays were 16.0% (low: 1.2ng/ml), 9.8% (mid: 3.4ng/ml) and 7.8% (high: 12.3ng/ml). Inter-assay c.o.v. were 16.8%, 8.1% and 13.2% for the three plasma pools respectively.

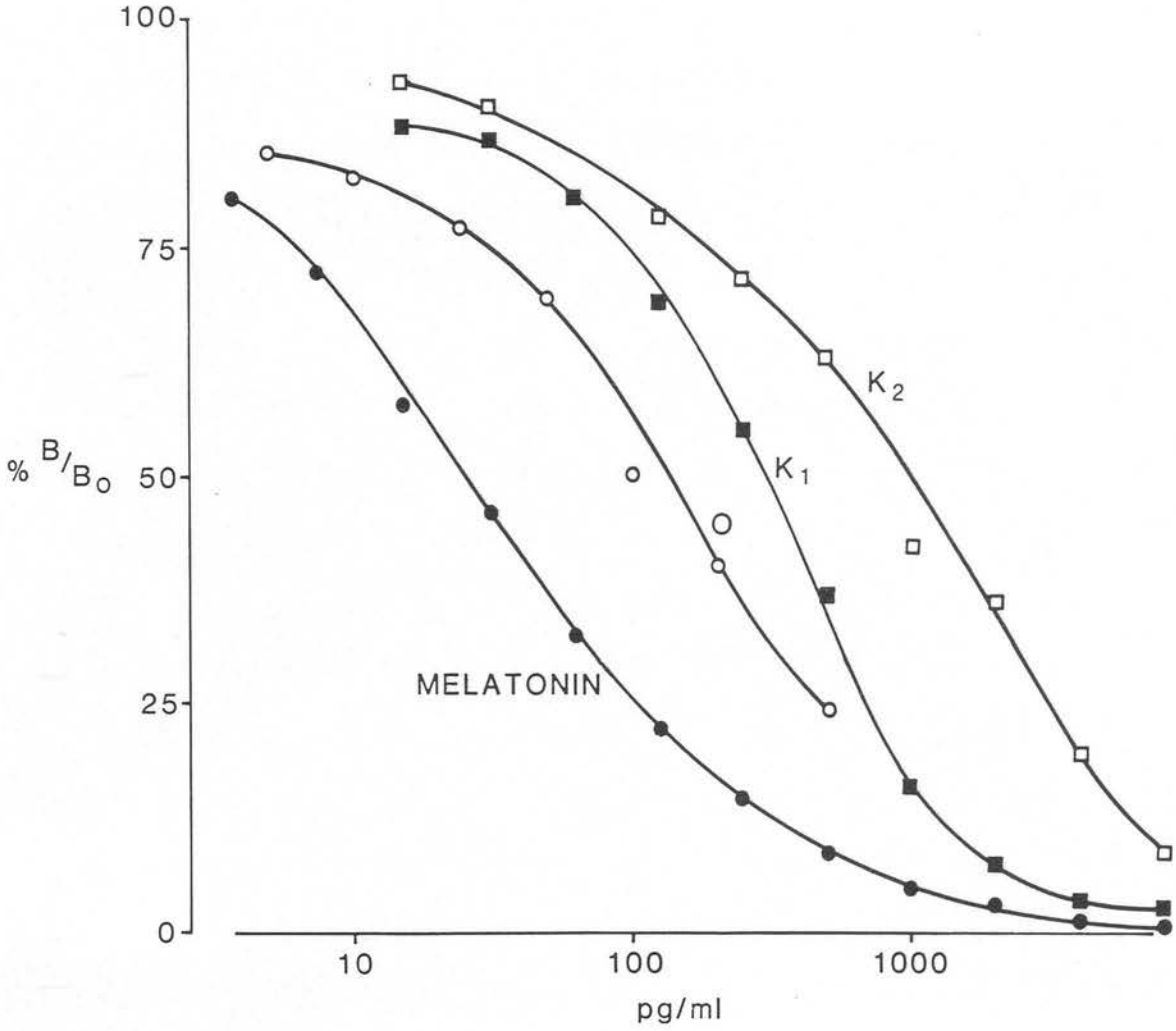

Fig. 2.21

Standard curves of melatonin (●), O-acetyl-5-methoxytryptophol ("O", O), N-acetyl-5-methoxykynurenamine ("K_2", □) and N-acetyl-N-formyl-5-methoxykynurenamine ("K_1", ■) with antiserum R1055.

2.4 Radioimmunoassay of Prolactin

Plasma prolactin concentrations were determined using the homologous ovin RIA described by McNeilly and Andrews (1974). Reference standard was NIH-P-S9 (NIAMDD, USA), and antiserum R2532 was used at a final dilution of 1:330,000. Lower limit of detection was 1ng/ml. Intra-assay c.o.v. for duplicates of quality control plasma pools were 8.0% (low: 11.0ng/ml), 2.4% (mid: 49.1ng/ml) and 9.7% (high: 121ng/ml), and inter-assay c.o.v. for these plasma pools were 11.0%, 10.7% and 12.2% respectively.

2.5 Radioimmunoassay of Melatonin

Plasma melatonin concentrations were measured using the assay described by Rollag and Niswender (1976) as modified by Almeida (1982). Reference standard was obtained from Sigma (M5250), and antiserum R1055 was used at a final dilution of 1:256,000. Lower limit of detection was 10pg/ml. Extraction efficiency was measured by recovery of ^{3}H-melatonin added to quality control plasmas; duplicate recoveries were inserted in the assay every 80 tubes. Mean recovery was 84.8 $\pm$ 1.29% in 38 assays. Intra-assay c.o.v. based on duplicates of quality control plasma pools were 15.8% (low:65pg/ml) and 12.0% (high: 250pg/ml), and inter-assay c.o.v. for these plasma pools were 26.6% and 14.6% respectively.

Three compounds which were not tested by Rollag and Niswender (1976) when originally investigating the specificity of antiserum R1055 were checked for crossreactivity during the course of routine melatonin assays. O-acetyl-5-methoxytryptophol was kindly given by Dr R.F. Seamark (Adelaide), and N-acetyl-5-methoxykynurenamine and N-acetyl-N-formyl-5-methoxykynuremamine by Dr R.W. Kelly (Edinburgh). Figure 2.21 shows the displacement of binding of ^{125}I-melatonin analogue to R1055 in comparison with the melatonin standard. Table 2.22 shows relative poten cies calculated according to the formula of Rollag and Niswender as pg melatonin/pg trial compound at 50%

Table 2.22

Cross-reactivity of various compounds with the R1055 melatonin antiserum

COMPOUND	RELATIVE POTENCY
Melatonin	1.0
O-acetyl-5-methoxytryptophol	1.6×10^{-1}
N-acetyl-5-methoxykynurenamine	2.6×10^{-2}
N-acetyl-N-formyl--5-methoxykynurenamine	8.6×10^{-2}

displacement. Although these kynurenamines may be present in brain tissue as metabolites of melatonin, there is no evidence that they occur in significant amounts in plasma (Hirata et al., 1974; Kelly et al., 1984). Likewise O-acetyl-5-methoxytryptophol has not been identified in extracted plasma (O.F.X. Almeida, pers. comm.).

2.6 Radioimmunoassay of FSH

FSH was measured in plasma by the RIA system of McNeilly et al. (1976). Reference standard was NIH-FSH-S10, and antiserum M94 was used at a final dilution of 1:32,000. Lower limit of detection was 10ng/ml. Mean intra-assay c.o.v. based on low, medium and high quality control plasma pools was 12.3% and mean inter-assay c.o.v. was 14.7%. All the FSH assays were carried out by Mrs L. Downey.

2.7 Animals

Except where stated, all studies were carried out on rams of the Soay breed which were obtained either from P. Mapson (Cambridge) or bred form the existing flock at the Dryden Field Station. In the experiments using artificial photoperiods, rams were housed indoors in light proof sheds. The rams were penned individually, eight per room but were in visual and olfactory contact with each other. They were fed a pelleted concentrated diet (AA6, ABRO). This was available ad libitum from hoppers. The weight of food required to keep the hoppers full was recorded by Miss N. Anderson. Water was available ad libitum. Artificial daylight was provided by three fluorescent strip lights. Lights "on" and "off" were controlled by time clocks (Sangamo Western Ltd., Enfield). Changes from one photoperiod to another were made by altering the time of lights off. Light intensity (as measured by a Weston lightmeter) was approximately 160 lux at the rams' eye level. Throughout the study a 15W red bulb was used during the artificial night, to aid taking blood samples in the dark. Temperature was not controlled or measured, but heating was supplemented in winter to reduce the fluctuations. Testis diameter and inguinal sexual

skin flush were recorded biweekly using the method of Lincoln and Davidson (1977). Observations of moulting and horn growth were also made. Blood samples for hormone assays were collected weekly, between two and four hours after lights on.

Rams maintained under natural photoperiods were housed together in an outside paddock at the Dryden Field Station, ABRO, Roslin, Scotland (56°N). Diets were supplemented with hay and turnips in winter. As part of a concurrent study, some of the rams had been pinealectomized or ganglionectomized shortly after birth under fluorothane anaesthesia (Lincoln and Forbes, 1984). Blood samples were collected weekly. Body weight, testis diameter and sexual flush were measured biweekly by Dr G.A. Lincoln.

2.8 Collection of blood samples

Weekly samples were collected by jugular venepuncture, using a heparinized evacuated 7ml glass tube and a 21G gauge "vacutainer" needle (Beckton and Dickinson). Where serial samples were required at an interval of one hour of less, polythene cannulae (Braunula luer, Armour Pharmaceuticals) were inserted into the jugular vein of the rams on the day prior to the start of sampling and stitched in place with sutures through the skin of the neck. This did not require anaesthesia. The cannulae were connected to three way taps (Vygon-VG1) by 60cm polythene tubes (Portex) and kept patent with 0.9% saline (Travenol Labs.) containing 25000 IU heparin/litre (Weddel Pharmaceuticals). Blood samples were collected into 5ml or 10ml plastic syringes (Plastipak), transferred to heparinised glass tubes, and centrifuged for 20 minutes at 2000 rpm. Plasma was removed and stored in capped 5ml vials (Sardstedt) at -20°C prior to assay. Blood samples collected for studies for plasma β-endorphin-like immunoreactivity were treated as described previously.

2.9 Statistical analysis

LH pulses

Plasma LH concentrations for individual rams in the experiments described in chapter 3 were analysed using a modification of the method described by Fraser and Lincoln (1980). A significant LH pulse was defined as two consecutive "peak" values exceeding two previous consecutive "baseline" values, with one of the peak values exceeding the mean of the baseline values by three times the assay coefficient of variance (c.o.v.). In experiments 1 to 4 the intra-assay c.o.v. was used in these calculations. This was derived from duplicates of three quality control pool plasmas with approximate values of 1.1, 4.3 and 11.2 ng/ml. For experiments 5-7 a computer programme was developed which used a moving c.o.v.. This was calculated using the duplicates of each successive group of four values that was tested for the existence of a significant pulse. Amplitude was calculated as the difference between the higher of the two peak values and the mean of the two baseline values. This program was written in BASIC and ran on a Commodore 4032 microcomputer.

Melatonin peaks

The hourly melatonin data for individual animals in the experiments in chapter 5 were analysed for peaks using a method developed with the assistance of Dr P. Warner. The analysis consisted of the following steps:

1) The data for each sampling period for each ram were ranked, and divided into classes of 10, 15 or 20 pg/ml thus giving a frequency distribution for melatonin values.

2) The assumption was made that under conventional light-dark cycles the distribution of melatonin values would be bimodal, ie. there would be a distinct group of "night-time" values separate from a group of

"day-time" values. From the frequency distribution the position of the discontinuum between night-time and day-time levels was obtained. Where no discontinuum was observed the median value was obtained.

3) A "runs test" (Siegel, 1956) was performed on each data set to determine whether there was significant clustering of the melatonin values above and below the chosen discontinuum or median. Where no significant clustering was observed the data were considered to be non-rhythmic.

4) Where significant clustering was indicated, actual melatonin peaks were defined as those containing four or more consecutive values above the discontinuum/median, the criterion adopted by Almeida and Lincoln (1982). Shorter runs separated from a peak of four or more values by a single low value were considered to be an extension of the same peak.

5) Onset of the peak was defined as the time of the first value above the discontinuum/median, and decline was taken to be the time of the first value following the defined peak below the discontinuum/median value. Duration was defined as the time from the onset to the decline, and period was defined as the length of time between onsets of successive peaks.

Other analyses

Preliminary processing of data was carried out on a Sirius microcomputer using the "Simplestat" data handling package written by Dr. P. Warner. All analyses of variance (ANOVAR) were performed using the methods described by Winer (1971). These were either calculated by hand, or by programs written in BASIC and run on a Commodore 4032 microcomputer. Full details of the particular ANOVAR used and of subsequent significance testing are given where appropriate.

Chapter 3

Effects of opiate agonists and antagonists on LH and prolactin secretion

3.1 Experiment 1 Effects of morphine on pulsatile LH release in sexually active intact and pinealectomized rams.

3.1.1 Aims

As reviewed in chapter 1.2.4, many studies in rats and man have demonstrated that alkaloid opiates suppress plasma LH levels, though few studies have investigated which parameters of LH secretion were altered. In sheep certain anaesthetics have been shown to suppress tonic LH release, for example an etorphine/acepromazine mixture (Peet and Lincoln, 1977), and halothane following thiopentone induction (Clarke and Doughton, 1983). The aim of this experiment was to investigate whether specific opiate mechanisms might be involved in the central suppression of tonic LH secretion by studying the effects of morphine on pulsatile LH secretion in rams and the effects of simultaneous administration of the specific opiate antagonist naloxone.

3.1.2 Materials and Methods

Twelve 2½ year old Soay rams normally kept in outside paddocks were used in this study. Six were intact, and six had been pinealectomized shortly after birth. The study was carried out in mid-October, the peak in testicular activity in the intact rams, and a period when the pinealectomized rams are also still showing full reproductive activity (Lincoln and Forbes, 1984). The rams were temporarily housed indoors but received natural daylight through large windows.

Jugular cannulae were inserted the day prior to study, and serial blood samples were collected every 15 minutes for 10 hours on two successive days. Sampling started at 08.00 on each day. On day two, six rams each received 20mg morphine sulphate (Pharmacy, RIE), administered i.v. via the jugular cannulae as four 5mg injections at hourly intervals,

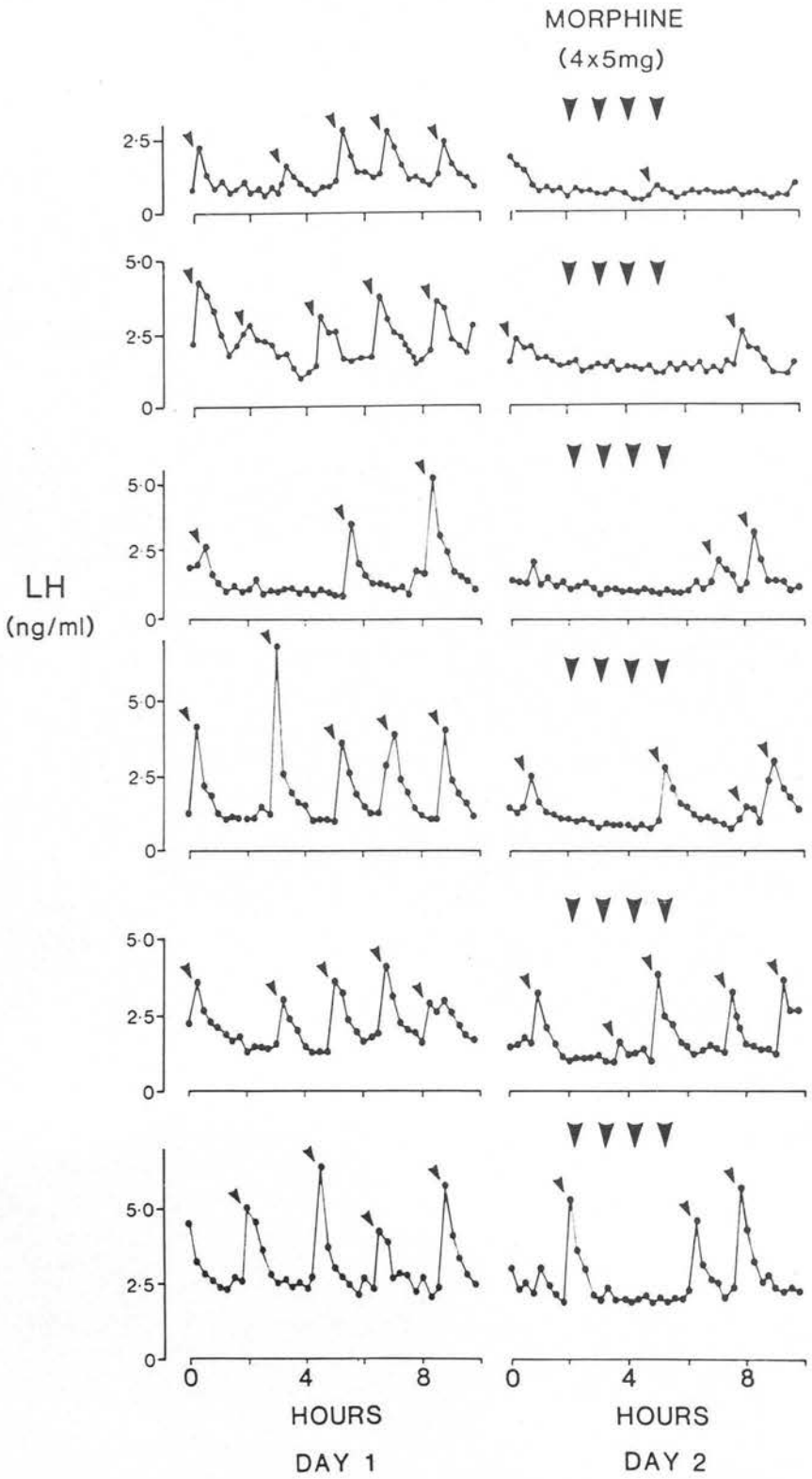

Fig. 3.1.1

Experiment 1. Effects of morphine on plasma LH levels in six Soay rams in October. Each ram received 20mg morphine i.v., given as 4 x 5mg doses at hourly intervals on day 2. Significant LH pulses are indicated by ▼.

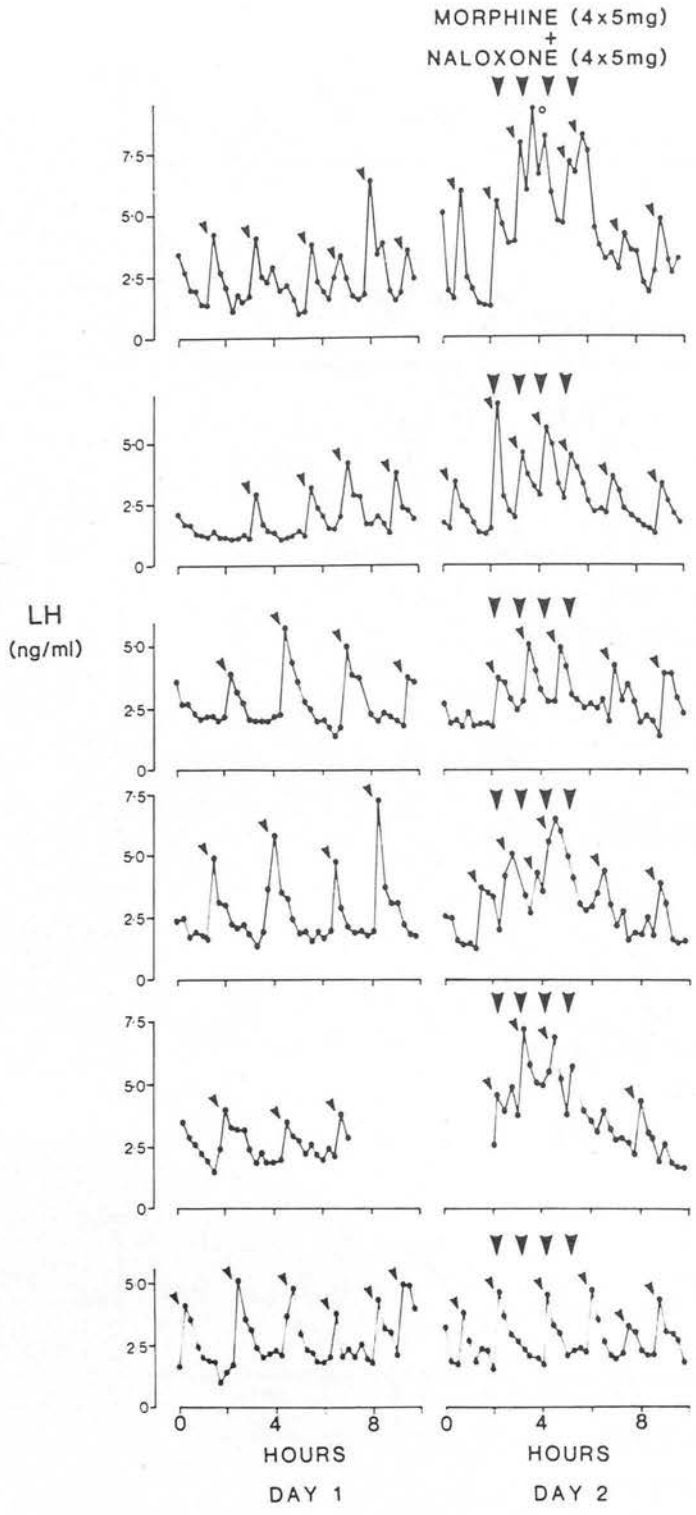

Fig. 3.1.2

Experiment 1. Effects of morphine plus naloxone on plasma LH levels in six Soay rams in October. Each ram received 20mg morphine i.v., and 20mg naloxone i.v. on day 2. These were given concurrently as 4x5mg doses at hourly intervals. Significant LH pulses are indicated by ▼.

Table 3.1.3

Experiment 1 : Effects of morphine (20mg) or morphine (20mg) + naloxone (20mg) on LH secretion

		OVERALL LH LEVEL (ng/ml)		LH PULSE FREQUENCY (pulse/6h)		LH PULSE AMPLITUDE (ng/ml)	
		DAY 1 CONTROL	DAY 2 TREATMENT	DAY 1 CONTROL	DAY 2 TREATMENT	DAY 1 CONTROL	DAY 2 TREATMENT
INTACT	MORPHINE (n=3)	2.43 ± 0.35	1.79 ± 0.43	2.95 ± 0.21	2.33 ± 0.33	2.7 ± 0.42	1.99 ± 0.50
	MORPHINE + NALOX (n=3)	2.42 ± 0.29	3.12 ± 0.16	2.95 ± 0.42	4.33 ± 0.33	2.37 ± 0.24	2.34 ± 0.15
	COMBINED (n=6)	2.42 ± 0.20		2.95 ± 0.21		2.53 ± 0.23	
PINX	MORPHINE (n=3)	1.65 ± 0.32	1.00 ± 0.21	2.74 ± 0.42	1.0 ± 0.0	1.74 ± 0.19	0.83 ± 0.18
	MORPHINE + NALOX (n=3)	2.53 ± 0.08	4.46 ± 0.50	3.19 ± 0.36	4.66 ± 0.66	2.85 ± 0.66	2.24 ± 0.18
	COMBINED (n=6)	2.09 ± 0.25		2.96 ± 0.27		2.30 ± 0.39	
INTACT + PINX	MORPHINE (n=6)	2.04 ± 0.27	1.40 ± 0.28***	2.84 ± 0.22	1.66 ± 0.33**	2.22 ± 0.30	1.41 ± 0.35*
INTACT + PINX	MORPHINE + NALOX (n=6)	2.48 ± 0.14	3.79 ± 0.38**	3.07 ± 0.25	4.50 ± 0.34***	2.61 ± 0.33	2.29 ± 0.11

Values are mean ± S.E.M. Asterisks indicate significant differences from the respective control period:
* $p<0.05$ ** $p<0.025$ *** $p<0.01$

commencing two hours after the start of blood sampling. The morphine was dissolved in 0.9% sterile saline, each injection volume being 2.5ml. The other six rams were given 20mg morphine sulphate and 20mg naloxone hydrochloride (Sterling Winthrop, UK) on day two, the latter also being dissolved in the 0.9% saline vehicle. Each was administered i.v. as four 5mg doses, the naloxone being given approximately five minutes before the morphine. Plasma was collected, and LH assayed as previously described.

3.1.3 Results

Fig. 3.1.1 illustrates the individual plasma LH concentrations in rams receiving morphine only on day 2, and Fig. 3.2.1 illustrates plasma LH profiles from the rams receiving both morphine and naloxone on day 2. Table 3.1.3 shows the overall plasma LH levels and the mean LH pulse frequencies and amplitudes. LH pulses are as previously defined in chapter 2. The day 2 treatment period is defined as being from the sample after the first injection to three hours after the last injection.

Students 't' tests indicated no significant differences in LH pulse frequency, amplitude or overall LH levels between intact and pinealectomized rams on the control day, therefore these groups were combined together to test the effect of treatments. Paired 't' tests indicated: 1) a very significant ($P<0.025$) decrease in LH pulse frequency during morphine treatment on day 2 as compared to the control day, and thus a highly significant ($P<0.01$) decline in overall LH levels, and 2) a very significant ($P<0.01$) increase in LH pulse frequency during combined naloxone and morphine treatment on day 2 as compared to the control day, and a corresponding significant ($P<0.025$) increase in overall LH levels. No significant change in pulse

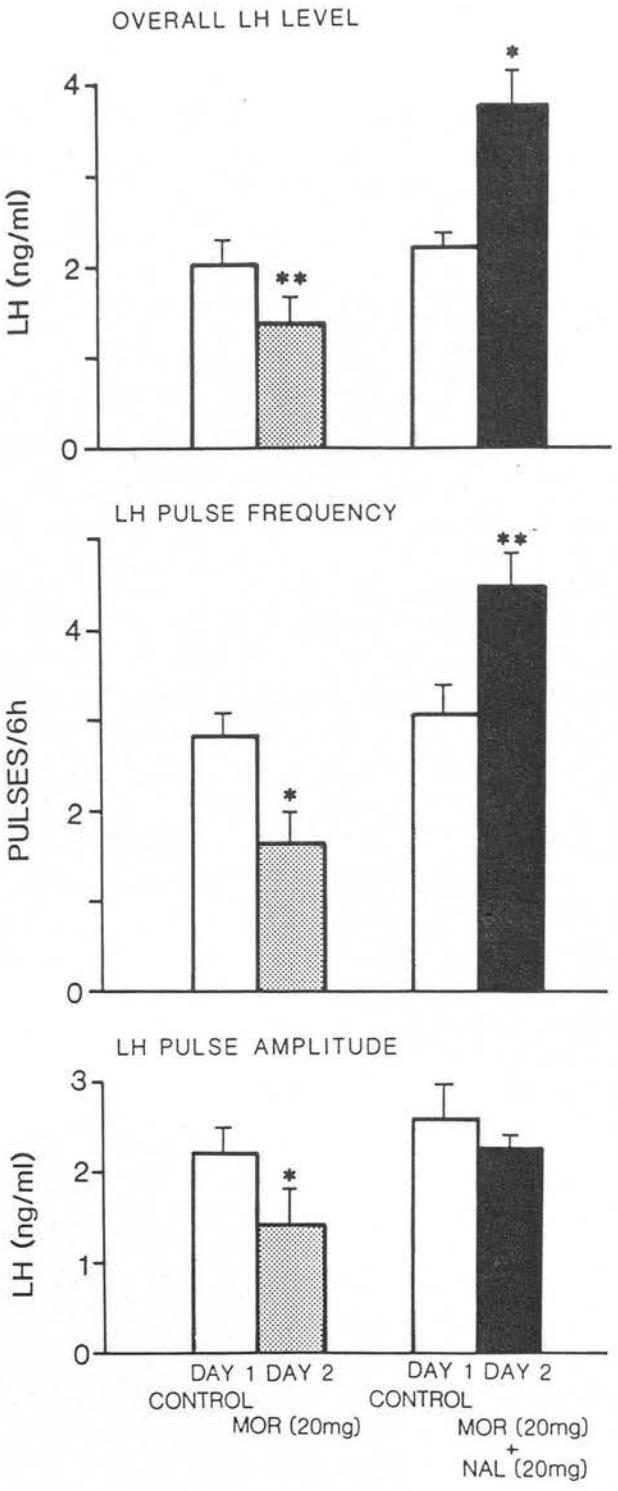

Fig. 3.1.4

Experiment 1. Summary of effects of morphine or morphine plus naloxone on LH secretion in sexually active rams. Values for each group are mean ± S.E.M., n=6. Significant differences are indicated by asterisks : * $p<0.05$, ** $p<0.01$. See text for explanation of statistics.

amplitude resulted from naloxone plus morphine treatment. The data are summarised in Figure 3.1.4.

3.2 Experiment 2. Comparison of the effects of morphine and β-endorphin and the antagonistic effects of naloxone on plasma LH and prolactin concentrations in sexually active intact and SCGx rams

3.2.1 Aims

Various studies have indicated that peripheral (i.v.) administration of endogenous opioid peptides alter neuroendocrine function, for example β-endorphin increases prolactin and growth hormone secretion in rats (Dupont et al., 1977; Grandison and Guidotti, 1977; Rivier et al., 1977). The aim of this experiment was to investigate whether β-endorphin given peripherally would alter pulsatile LH release and prolactin secretion in sexually active rams, and to compare the response to that with the alkaloid opiate morphine. Naloxone was also given to investigate whether the effects of alkaloid and peptide opiates were equally reversible with an alkaloid opiate antagonist.

3.2.2 Materials and methods

This study was carried out in the first week of November using eight 2 year old Soay rams which were normally maintained in outdoor paddocks. Four were intact, and four had been previously superior cervical ganglionectomized (SCGx) shortly after birth (Lincoln and Forbes, 1984). The rams were temporarily housed indoors and jugular cannulae were inserted on the day prior to study. On two subsequent

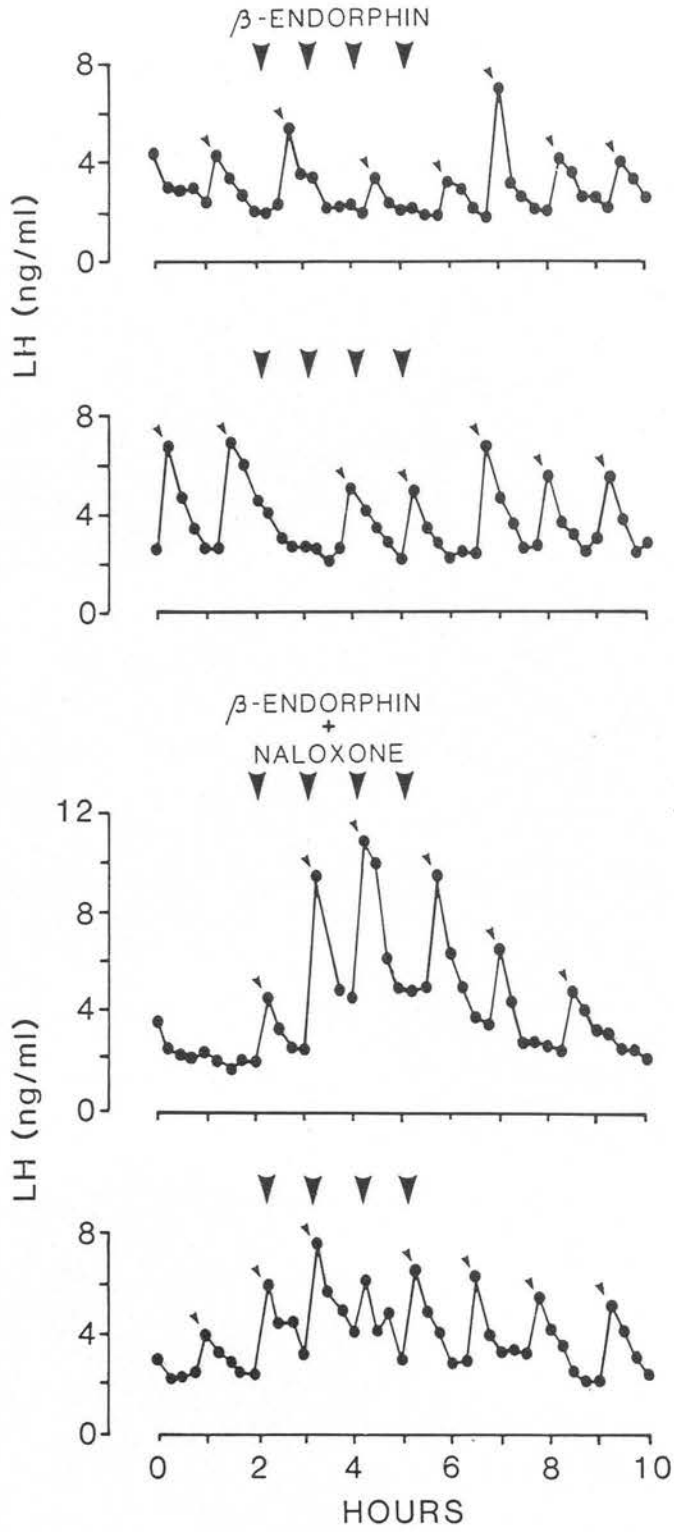

Fig. 3.2.5a

Experiment 2. Effects of β-endorphin on plasma LH levels in individual Soay rams in early November. Significant LH pulses are indicated by ▼. The upper profiles are from two rams which received 200μg $\beta_o$endorphin i.v. given as 4x50μg injections at hourly intervals. The lower profiles are from two rams which received 200μg $\beta_h$endorphin plus 8mg naloxone. These were given concurrently as 4x[50μg $\beta_o$endorphin + 2mg naloxone] at hourly intervals.

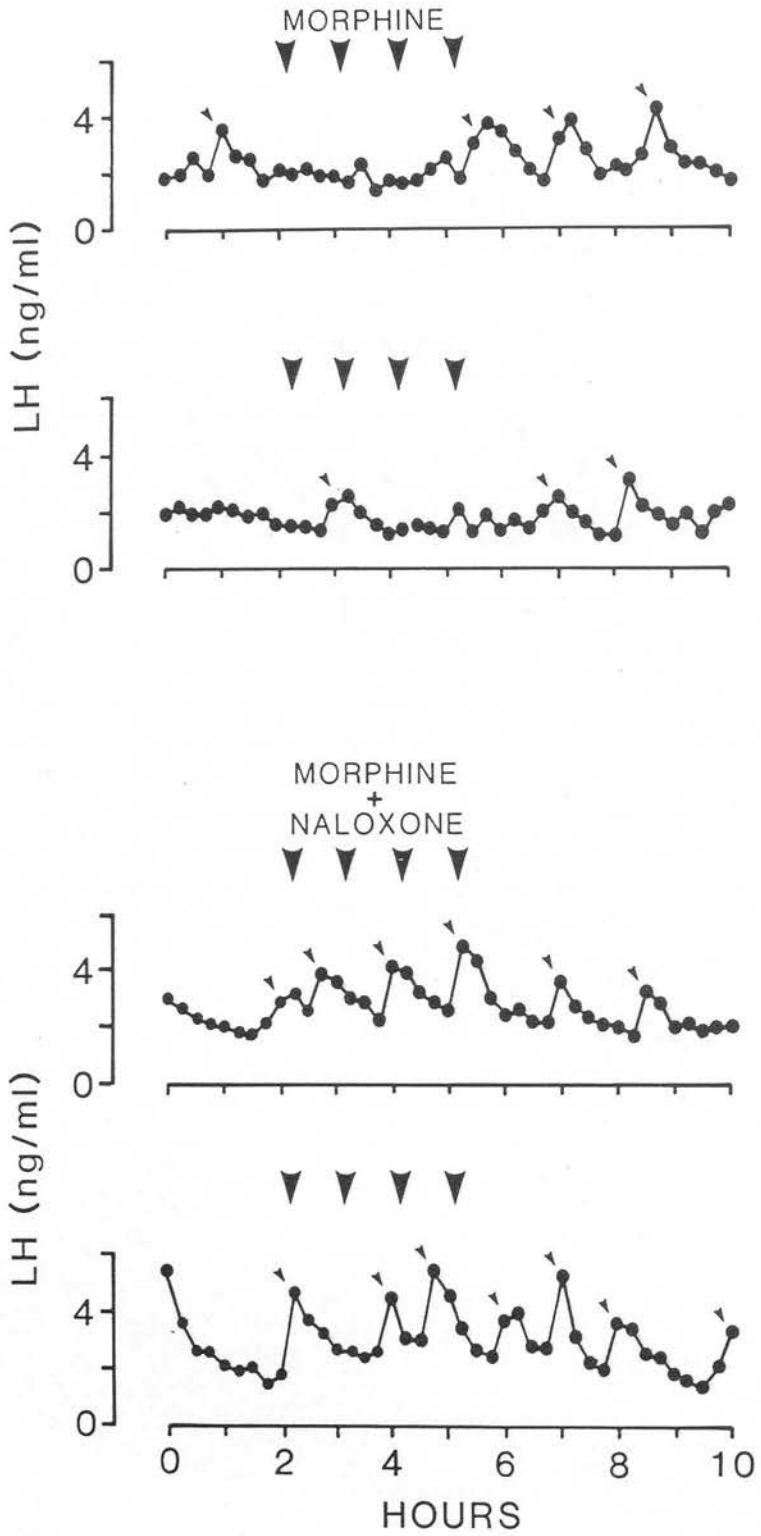

Fig. 3.2.5b

Experiment 2. Effects of morphine on plasma LH levels in individual Soay rams in early November. Significant LH pulses are indicated by ▼. The upper profiles are from two rams which received 8mg morphine i.v. given as 4x2mg injections at hourly intervals. The lower profiles are from two rams which received 8mg morphine plus 8mg naloxone. These were given concurrently as 4x[2mg morphine + 2mg naloxone] at hourly intervals.

Table 3.2.6

Effects of morphine(8mg) or β-endorphin(200ug) with or without naloxone(8mg) on LH secretion in sexually active Soay rams.

TREATMENT (n=4 per group)	OVERALL LH LEVEL ng/ml PRE-TREATMENT	OVERALL LH LEVEL ng/ml POST-TREATMENT	LH PULSE FREQUENCY PRE-TREATMENT 12h	LH PULSE FREQUENCY POST TREATMENT /6h	LH PULSE FREQUENCY POST TREATMENT /2h	LH PULSE AMPLITUDE ng/ml PRE-TREATMENT	LH PULSE AMPLITUDE ng/ml POST-TREATMENT
β-EP	3.38 ± 0.59	2.87 ± 0.17[ac]	1.25 ± 0.25	3.25 ± 0.75[c]	1.08	2.45 ± 0.88	2.72 ± 0.49
β-EP + NALOXONE	2.71 ± 0.17	4.47 ± 0.55[ad]	0.67 ± 0.33	5.67 ± 0.33[c]	1.89	2.70 ± 1.1	2.96 ± 0.96
MORPHINE	2.10 ± 0.08	1.94 ± 0.08[bc]	0.75 ± 0.25	2.25 ± 0.5[d]	0.75	1.25 ± 0.35	1.53 ± 0.17
MORPHINE + NALOXONE	2.46 ± 0.40	3.24 ± 0.18[bd]	0.75 ± 0.25	5.25 ± 0.48[d]	1.75	1.72 ± 0.83	1.84 ± 0.19
INTACT DAY1	2.87 ± 0.19		1.0 ± 0.00			2.32 ± 0.74	
SCGX DAY1	3.23 ± 0.65		1.0 ± 0.48			2.90 ± 1.3	
INTACT DAY2	2.45 ± 0.34		0.75 ± 0.25			1.18 ± 0.26	
SCGX DAY2	2.11 ± 0.23		0.75 ± 0.25			2.05 ± 0.45	

Values are mean ± S.E.M.
Values in the same column with the same subscripts differ significantly: a, b $p<0.01$; c, d $p<0.05$
See text for statistical methods.

days blood samples were collected at fifteen minute intervals for ten hours, starting at 08.00 on each day.

On day one, after a two hour pretreatment period four rams received 200ug synthetic ovine β-endorphin, given as 4 x 50ug i.v. injections at hourly intervals. The other four rams recieved $\beta_o$endorphin in the same manner, but additionally received 8mg naloxone hydrochloride (Du Pont, N.Y.), given as 4 x 2mg i.v. injections, each one just prior to the $\beta_o$endorphin injection. Both the $B_o$endorphin and the naloxone were dissolved in 0.9% saline, and each injection volume was made up to 2ml.

On day two the rams were reassigned to new treatment groups and the protocol was repeated using morphine sulphate instead of $\beta_o$endorphin. Each ram received a total dose of 8mg morphine as four 2mg i.v. injections, and half the rams also received four 2mg injections of naloxone, each given just prior to a morphine injection. Plasma was collected and LH and prolactin assayed as previously described.

3.2.3 Results

Fig. 3.2.5a-b illustrate plasma LH profiles from two rams in each treatment group. Table 3.2.6 shows the mean pre and post-treatment overall LH levels and LH pulse frequency and amplitude. The post treatment period was defined as being from the sample after the first treatment injection to three hours after the last of the four treatment injections.

The data were initially analysed for effects of ganglionectomy and day of experiment on pretreatment plasma LH parameters. One way ANOVAR indicated that there was no significant variance attributable to these effects on overall LH levels ($F=2.52$, ns), pulse frequency ($F=1.11$, ns)

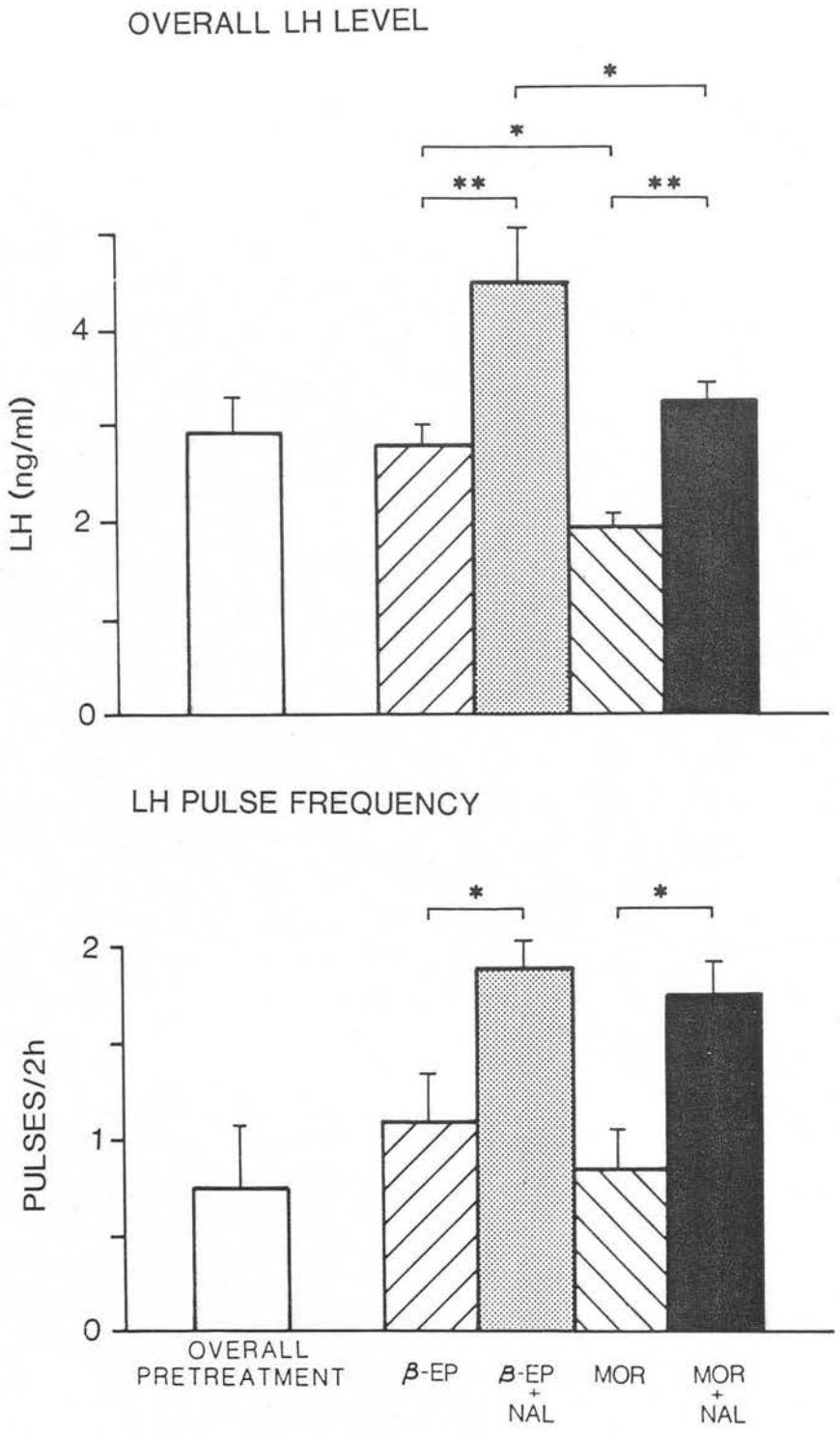

Fig. 3.2.7

Experiment 2. Summary of the effects of $\beta_o$endorphin or morphine treatment with or without concurrent naloxone administration on overall plasma LH levels and LH pulse frequency. Values for each treatment group are mean $\pm$ S.E.M., n=4. Significant differences are indicated by asterisks : * $p<0.05$, ** $p<0.01$. See text for explanation of statistics.

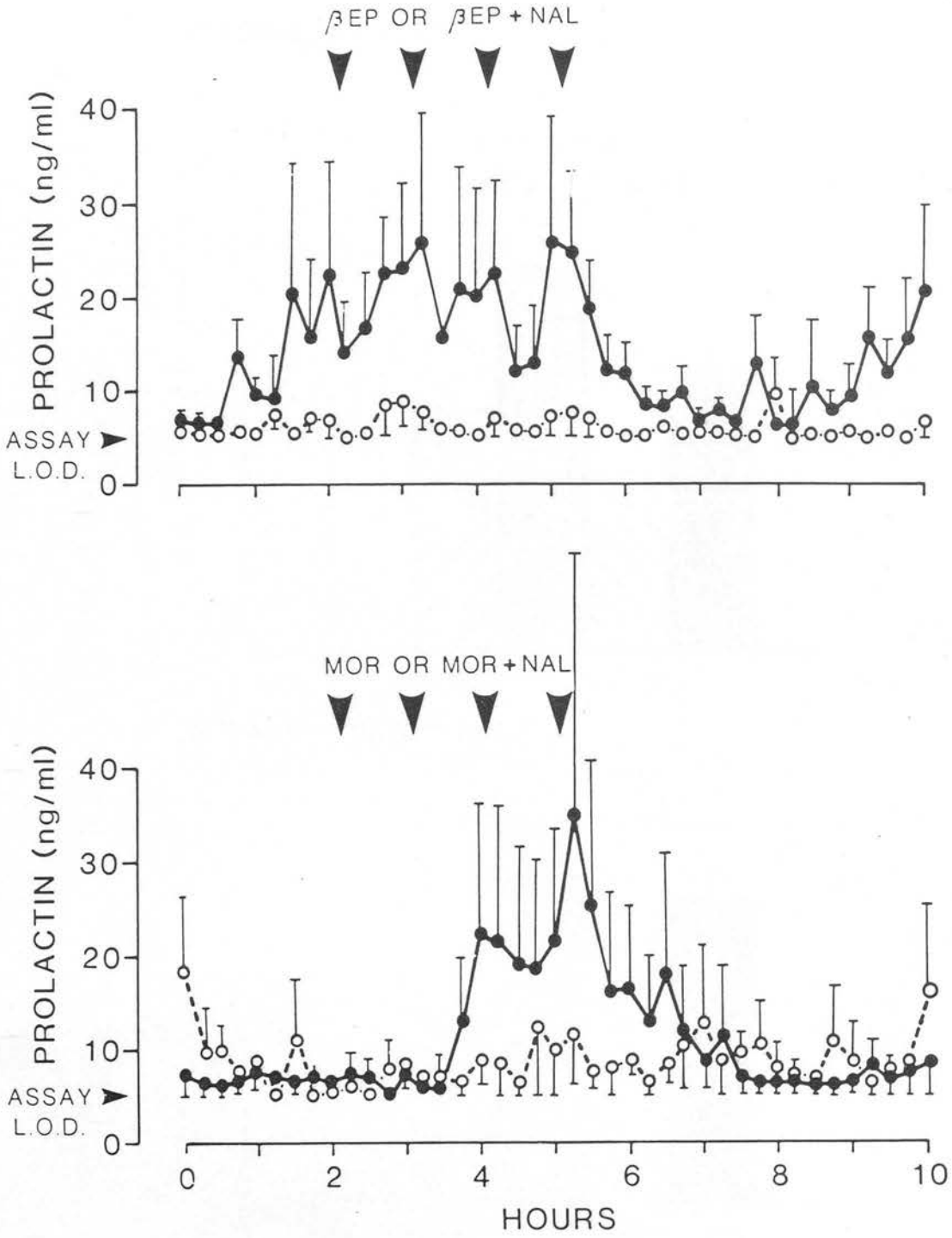

Fig. 3.2.8-9

Experiment 2. Effects of β-endorphin and morphine on plasma prolactin levels in Soay rams in early November. Values are mean ± S.E.M., n=4 for each treatment group. The upper panel (fig. 3.2.8) shows rams which either received a total dose of 200ug $\beta_o$endorphin (●) or 200ug $\beta_o$endorphin plus 8mg naloxone (O). The lower panel (fig. 3.2.9) shows rams which either received a total dose 8mg morphine (●) or 8mg morphine plus 8mg naloxone (O).

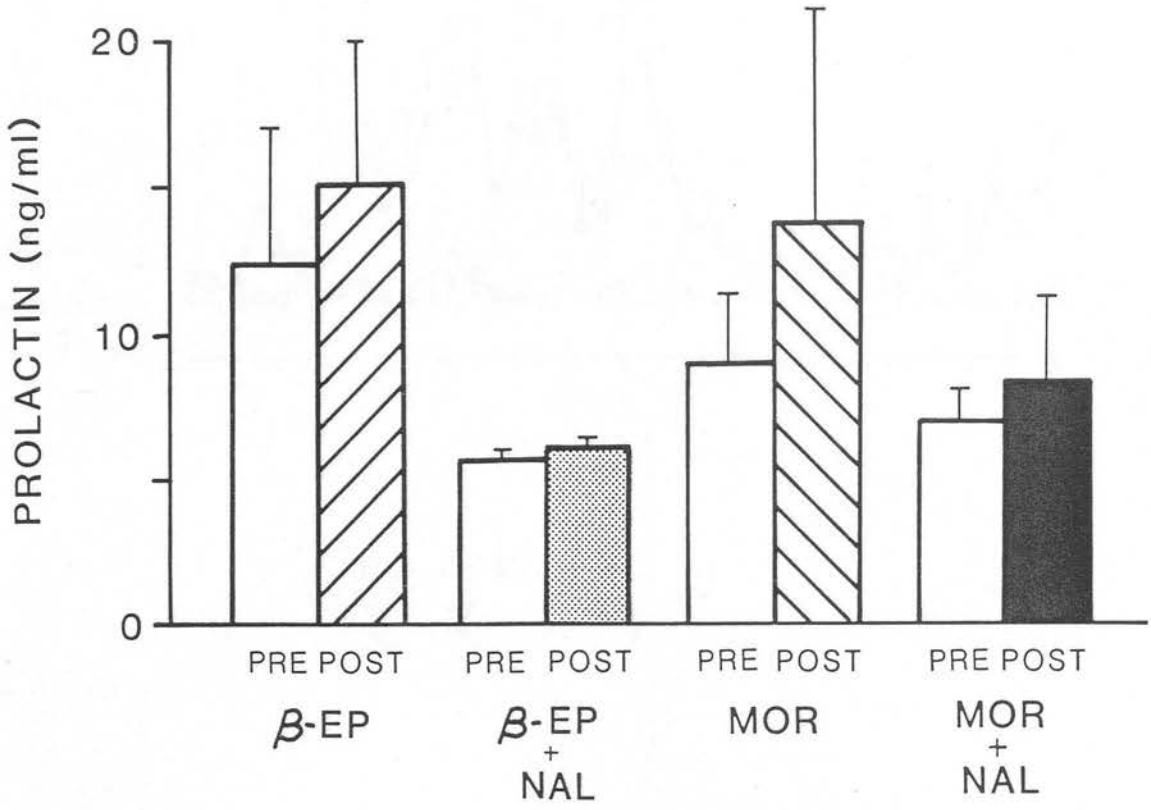

Fig. 3.2.10

Experiment 3. Summary of the effects of $\beta_o$endorphin or morphine treatment with or without concurrent naloxone administration on overall plasma prolactin levels. Values for each treatment group are mean $\pm$ S.E.M., n=4.

or pulse amplitude (F=1.44, ns). Data from intact and SCGx rams were therefore combined in subsequent analyses of effects of treatment.

One-way ANOVAR indicated no significant variance in any parameters of LH secretion between the various groups prior to treatment. Following the start of treatment highly significant variance was observed in both overall LH levels (F=15.89, $p<0.01$) and LH pulse frequency (F=7.23, $p<0.01$), though no significant variance in pulse amplitude occured. Significant differences between means of individual groups have been calculated using Duncan's New Multiple Range Test. This indicated that LH pulse frequency was significantly ($P<0.05$) higher in the $\beta_o$endorphin plus naloxone group than in the $\beta_o$endorphin only group, and likewise significantly higher ($P<0.05$) in the morphine plus naloxone group than in the morphine only group. These two comparisons were highly significant ($P<0.01$) for overall LH levels. Overall LH levels were also significantly lower ($P<0.05$) in the morphine only group compared to the $\beta_o$endorphin only group, and significantly lower in the morphine plus naloxone group than in the $\beta_o$endorphin plus naloxone. Fig. 3.2.7 summarises these results.

Figures 3.2.8 and 3.2.9 illustrate mean plasma prolactin levels in the four treatment groups. In the pre-treatment period prolactin levels were significantly higher in the SCGx rams compared to the intact rams (14.8 ± 2.73 vs 5.7 ± 0.38 ng/ml; $p<0.05$), the majority of the samples from intact animals being below the limit of detection of the assay. The data from each animal have therefore been normalised using their pre-treatment means in the subsequent analysis of effects of treatment. Fig. 3.2.10 summarises the pre- and post-treatment means for each treatment. There was no significant effect of treatment, however there appeared to be a trend towards greater

Table 3.3.11

Experiment 3: Summary of effects of human β-endorphin on plasma LH secretion and plasma prolactin levels in sexually inactive Soay rams.

	MEAN LH LEVEL ng/ml	LH PULSE FREQUENCY pulses/12h	LH PULSE AMPLITUDE ng/ml	MEAN PROLACTIN ng/ml
CONTROL (n=8)	0.71 ± 0.26	1.88 ± 0.40	2.00 ± 0.93	44.6 ± 7.62
$\beta_h$endorphin(125ug) (n=8)	0.77 ± 0.23	1.88 ± 0.40	2.45 ± 0.95	43.0 ± 4.53

Values are mean ± S.E.M.

increases in plasma prolactin levels following β-endorphin and morphine treatment than following concomit ant treatments with naloxone.

3.3 Experiment 3 Effects of human β-endorphin on plasma LH and prolactin concentrations in sexually inactive rams.

3.3.1 Aims

The design of experiment 2 did not permit a satisfactory comparison between plasma LH and prolactin levels in the $\beta_o$endorphin treatment period and data for a suitable control period. The aim of this preliminary study was to investigate in detail whether the peripheral administration of $\beta_h$endorphin had any effects on pulsatile LH secretion and plasma prolactin levels.

3.3.2 Materials and Methods

This study was carried out in April in sixteen one year old intact Soay rams which had been housed indoors on long days (16L:8D) for nine weeks prior to the experiment. Serial blood samples were collected by jugular cannulae at 20 minute intervals for twelve hours in all animals. Eight of the animals received 125ug synthetic human $\beta_h$endorphin (CRB Biochemicals, Cambridge) dissolved in 0.9% sterile saline. This was given as four x 30ug i.v. injections at hourly intervals, starting two hours after sampling commenced. The other eight control animals received only the saline vehicle.

3.3.3 Results

Table 3.3.11 summarises the results for LH pulse frequency and amplitude, and mean plasma LH and prolactin concentrations in control and treatment groups. No significant effects of $\beta_h$endorphin treatment on any of these parameters was observed.

3.4 Experiment 4 Preliminary studies of the effect of naloxone on plasma LH and prolactin concentrations in intact rams.

3.4.1 Aims

Studies in rats have indicated that naloxone given in i.v. doses of 2mg/kg b.w. alter meuroendocrine function (Ieiri et al., 1979; Meites et al., 1979). In humans effects of naloxone have been observed using lower i.v. doses of 0.1 - 0.2mg/kg b.w. (Morley et al., 1980; Quigley and Yen, 1980; Lightman et al., 1981a). The aim of this series of experiments was to investigate whether doses of naloxone of 0.05 - 0.1mg/kg b.w. would antagonize EOP mechanisms and therefore alter LH and prolactin secretion in rams.

3.4.2 Materials and Methods

Three preliminary studies were carried out to investigate the effects of low doses of naloxone on LH and prolactin secretion. Experiment 4a was carried out in 16 one year old intact Soay rams which had been housed indoors on an artificial "long day" light dark cycle (16L:8D) for seven weeks since mid-February. Blood samples were collected at 20 minute intervals for twelve hours. Eight rams received 1.6mg naloxone ("Narcan", Du Pont, U.K.) as 8 x 200ug i.v. injections at hourly intervals starting two hours after sampling commenced. The other eight rams received saline only as controls. Experiment 4b was carried out seventeen weeks later in the same rams which had subsequently been switched to short days (8L:16D) for twelve weeks prior to study. The experimental protocol was the same as in experiment 4a except that blood samples were collected at 15 minute intervals.

Table 3.4.12

Experiment 4: Effects of low doses of naloxone on plasma LH and prolactin concentrations in Soay rams.

	MEAN LH LEVEL (ng/ml)	LH PULSE AMPLITUDE (ng/ml)	LH PULSE FREQUENCY (pulses/12h)	MEAN PROLACTIN (ng/ml)
EXPT. 4a (WEEK 7, 16L:8D) CONTROL (n=8)	0.73 ± 0.04	2.51 ± 0.51	0.75 ± 0.25	26.2 ± 3.91
NALOXONE (n=8) (8x200ug)	0.92 ± 0.13	3.11 ± 0.70	1.63 ± 0.50	29.3 ± 3.62
EXPT. 4b (WEEK 12, 8L:16D) CONTROL (n=8)	1.96 ± 0.28	2.85 ± 0.47	6.5 ± 0.89	10.5 ± 2.54
NALOXONE (n=8) (8x200ug)	1.39 ± 0.26	1.97 ± 0.28	6.88 ± 0.55	10.5 ± 1.34
EXPT. 4c (WEEK 4, 16L:8D) CONTROL (n=4)	2.09 ± 0.11	1.22 ± 0.15	1.5 ± 0.29	102.6 ± 5.22
NALOXONE (1x2mg)	2.02 ± 0.16	1.54 ± 0.24	3.0 ± 0.71	74.4 ± 6.68

Values are mean ± S.E.M.

Experiment 4c was carried out in a group of 8 mature Soay rams which and been housed inside on alternating 16 week periods of short and long photoperiods for several years. At the time of study they were in week 5 of 16L:8D. Blood samples were collected at 20 minute intervals for twelve hours. Four of the rams received a single 2mg i.v. bolus of naloxone two hours after sampling started, the other four rams received saline only.

3.4.3 Results

Table 3.4.12 summarises the data from each experiment. Students 't' tests failed to reveal any significant effects of naloxone treatment, though there was a trend towards an increase in pulse frequency in the naloxone treated groups in experiments 4a and 4c.

3.5 Experiment 5 Effects of opiate antagonists on LH and prolactin secretion at different stages of the reproductive cycle in intact and non-pineal Soay rams.

3.5.1 Aims

There were three aims of this experiment;

1) To investigate whether higher doses of opiate antagonists than those used in experiment 4 would unequivocally demonstrate EOP mechanisms influencing LH and prolactin secretion in the ram

2) To investigate whether there were seasonal variations in the responses to opiate antagonists indicating seasonal changes in EOP-neuroendocrine interactions, and whether seasonal variations would exist in rams which were rendered non-photoperiodic by disruption of pineal melatonin secretion.

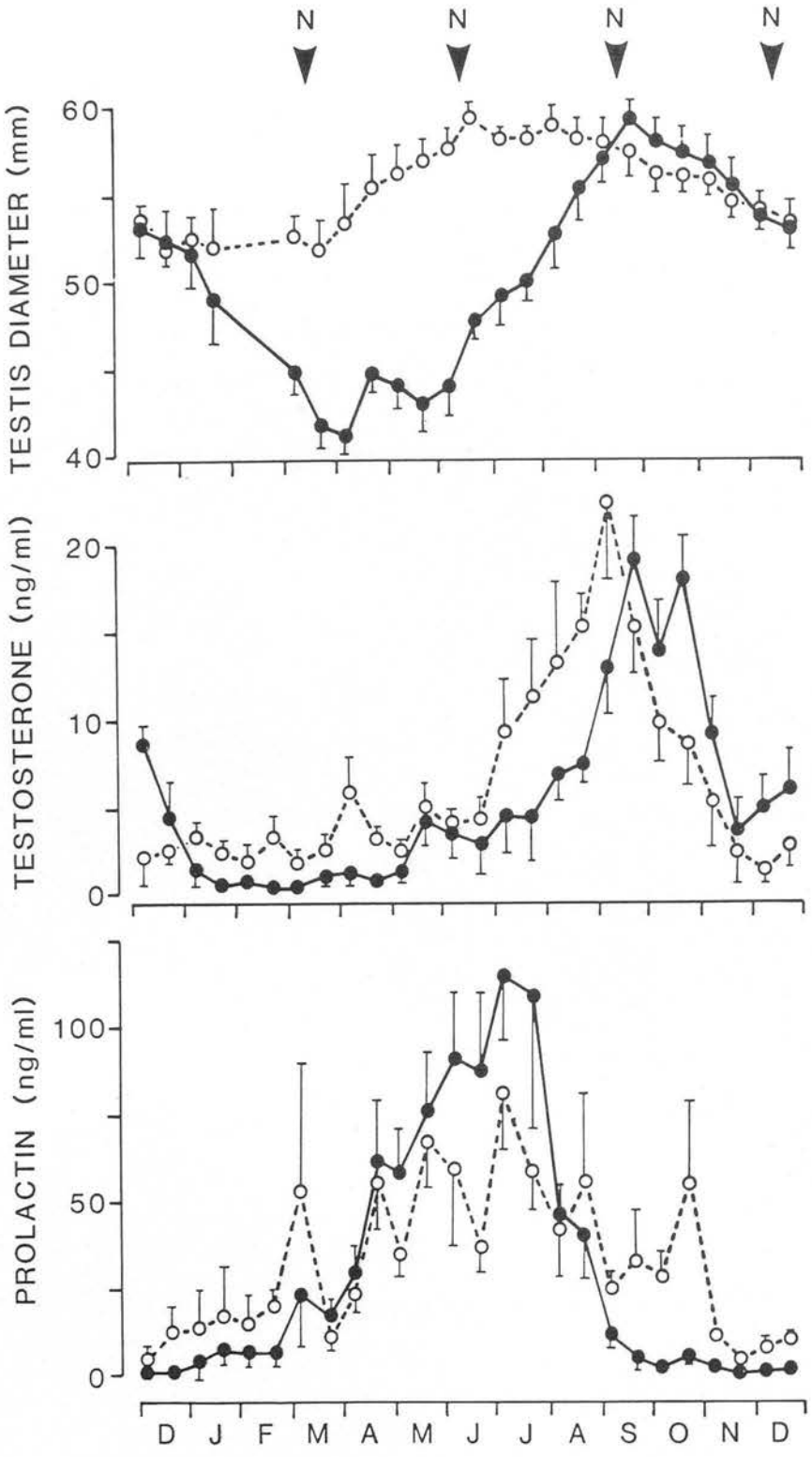

Fig. 3.5.13

Experiment 5. Annual cycles in testicular diameter, plasma testosterone an plasma prolactin levels in intact rams (●) and non-pineal rams (O) kept outside. Values for each group are mean ± S.E.M., n=3 for the intact group, and n=5 for the non-pineal group.
▼ indicates the times of year when responses to naloxone treatment were investigated.

3) To compare the responses to two different "pure" opiate antagonists (naloxone and naltrexone) and a mixed agonist-antagonist (diprenorphine)

3.5.2 Materials and methods

This study was carried out using twelve Soay rams of either two or three years age at the start of the experiment in March. Seven of the rams (the "non-pineal" group) had been pinealectomized (PINx) or superior cervical ganglionectomized (SCGx) shortly after birth, the remainder were intact. The animals were kept outside throughout the study except for the intensive blood sampling periods when the animals were brought indoors but maintained on the natural photoperiod.

This study was carried out in conjunction with a long term study of the seasonal physiology of these intact and non-pineal rams maintained outdoors (Lincoln and Forbes, 1984).

On four occasions (March, June, September and December) serial blood samples were collected at 20 minute intervals for 12 hours on two successive days using jugular cannulae inserted on the day prior to study. No treatment was given to any of the rams on day one. On day two, groups of four rams received naloxone hydrochloride (Endo labs, N.Y.), naltrexone hydrochloride (Endo labs, N.Y.) or diprenorphine hydrochloride ("Revivon", Reckitt and Colman, Hull, U.K.). Naloxone and naltrexone were both given at a total dose of 125mg/animal dissolved in 0.9% saline. This dose was 4-6mg/kg body weight. The naloxone/naltrexone was injected i.v. as four injections at hourly intervals. The first injection of 50mg was given two hours into the sampling period on day two, and three 25mg injections were subsequently given. A total dose of 25mg diprenorphine was given, in a similar protocol to that for the other antagonists ie. a 10mg injection

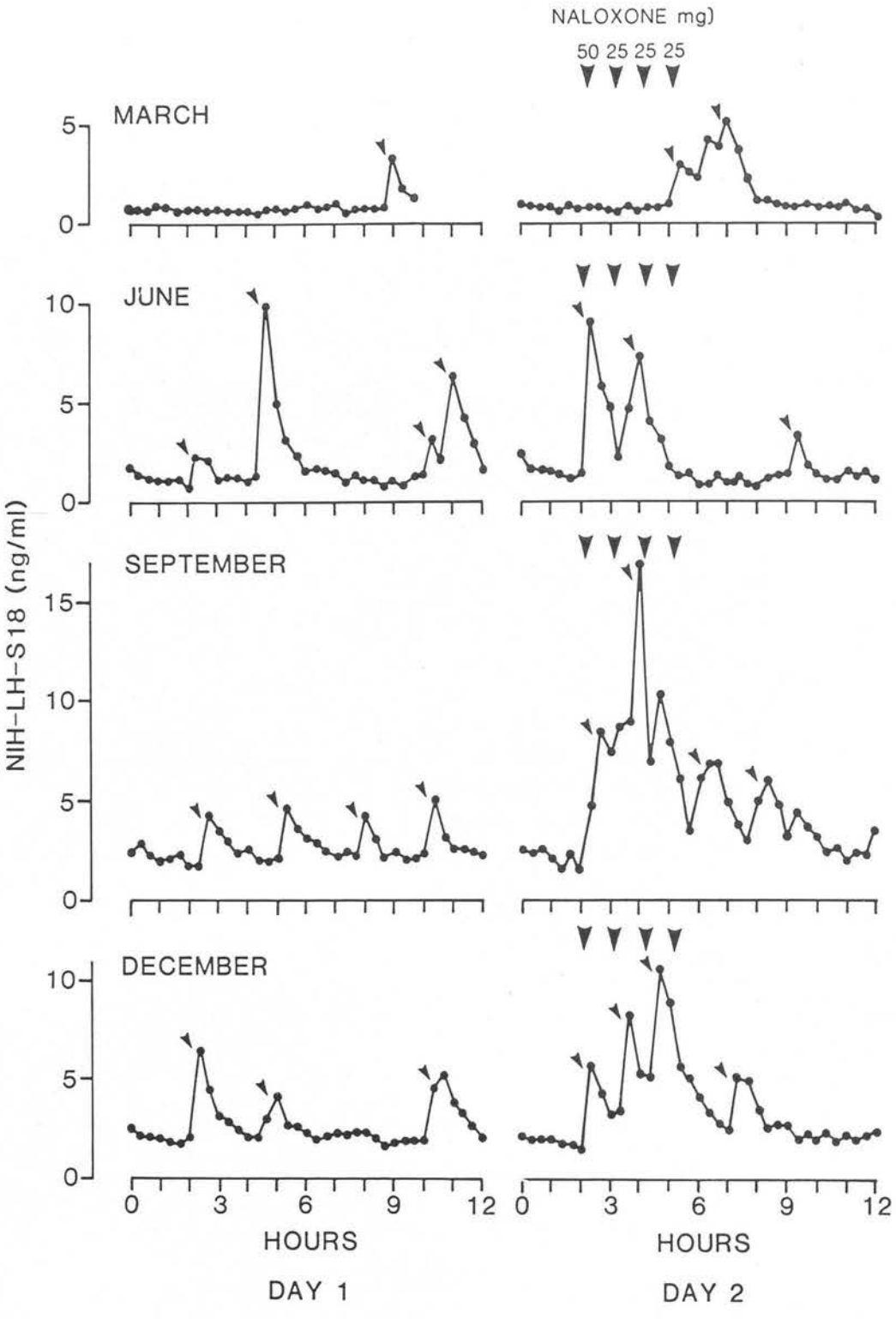

Fig. 3.5.14a

Experiment 5. Effects of naloxone on plasma LH concentrations at four times of year in an intact Soay ram living out-of-doors. On each occasion, day 1 was a control period, and on day 2 a total dose of 125mg naloxone was given i.v. over 4 hours. ▼ indicates a significant LH pulse.

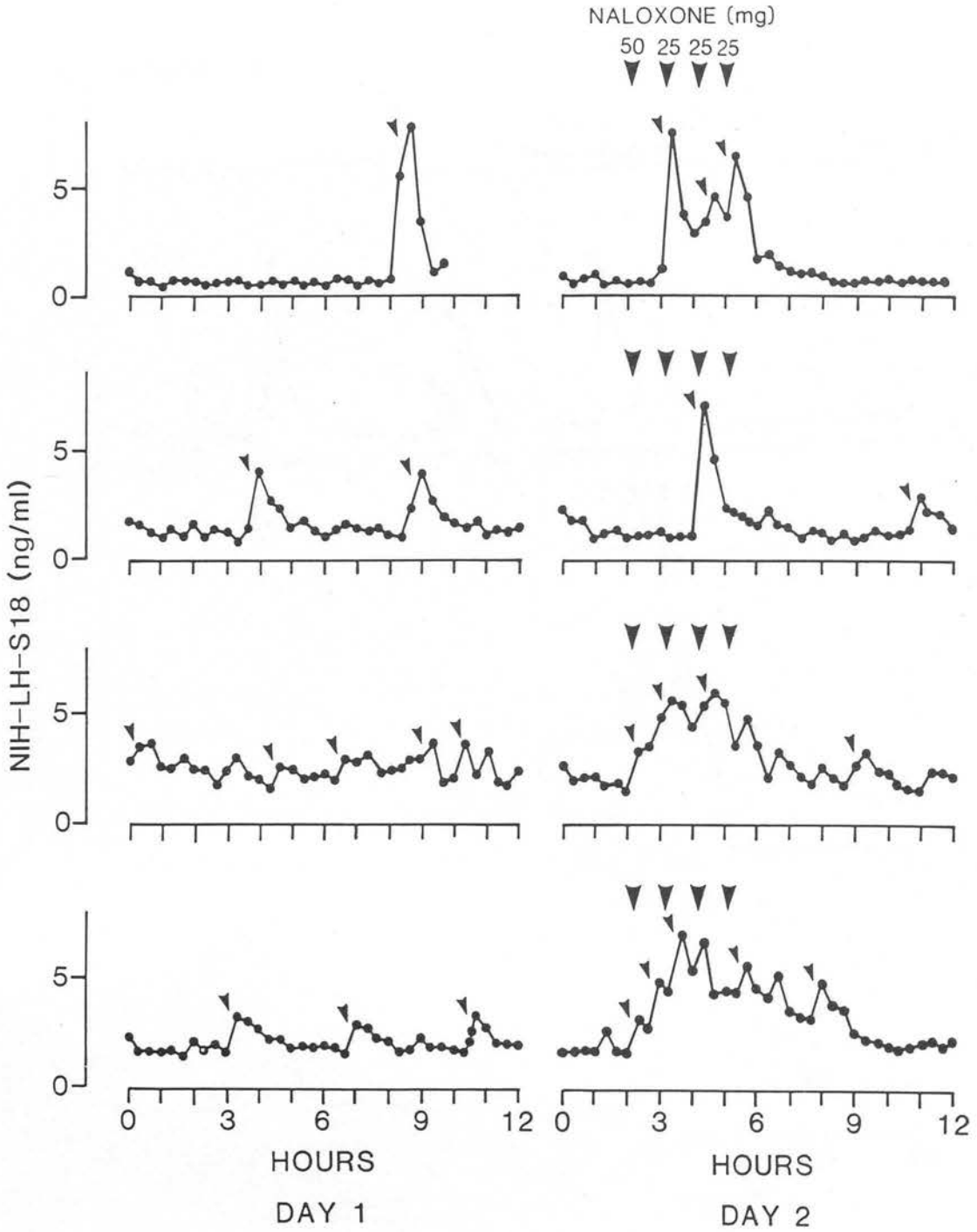

Fig. 3.5.14b

Experiment 5. Effects of naloxone on plasma LH concentrations at four times of year in an intact Soay ram living out-of-doors. On each occasion, day 1 was a control period, and on day 2 a total dose of 125mg naloxone was given i.v. over 4 hours. ▼ indicates a significant LH pulse.

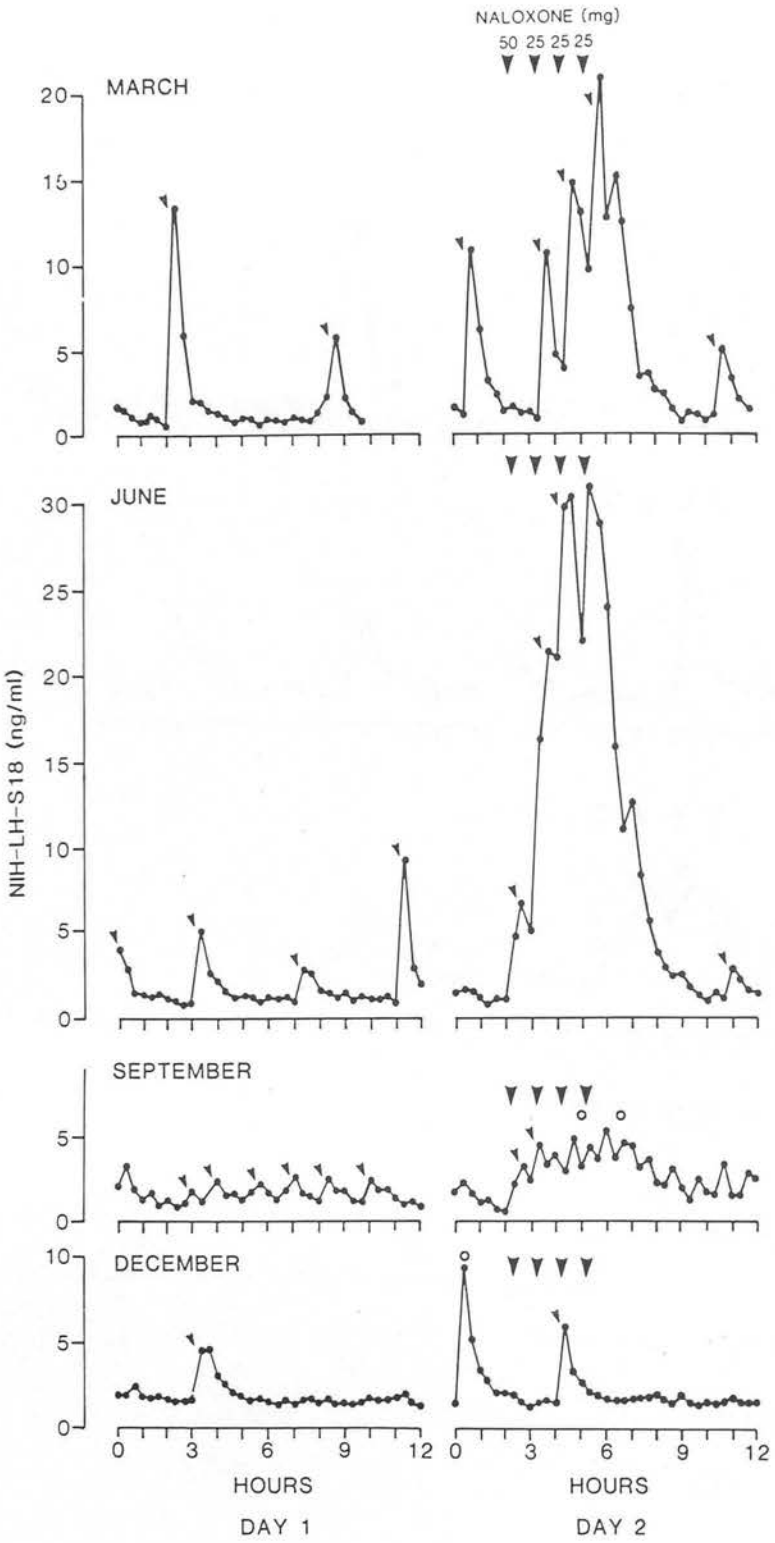

Fig. 3.5.14c

Experiment 5. Effects of naloxone on plasma LH concentrations at four times of year in a SCGx Soay ram living out-of-doors. On each occasion, day 1 was a control period, and on day 2 a total dose of 125mg naloxone was given i.v. over 4 hours. ▼ indicates a significant LH pulse.

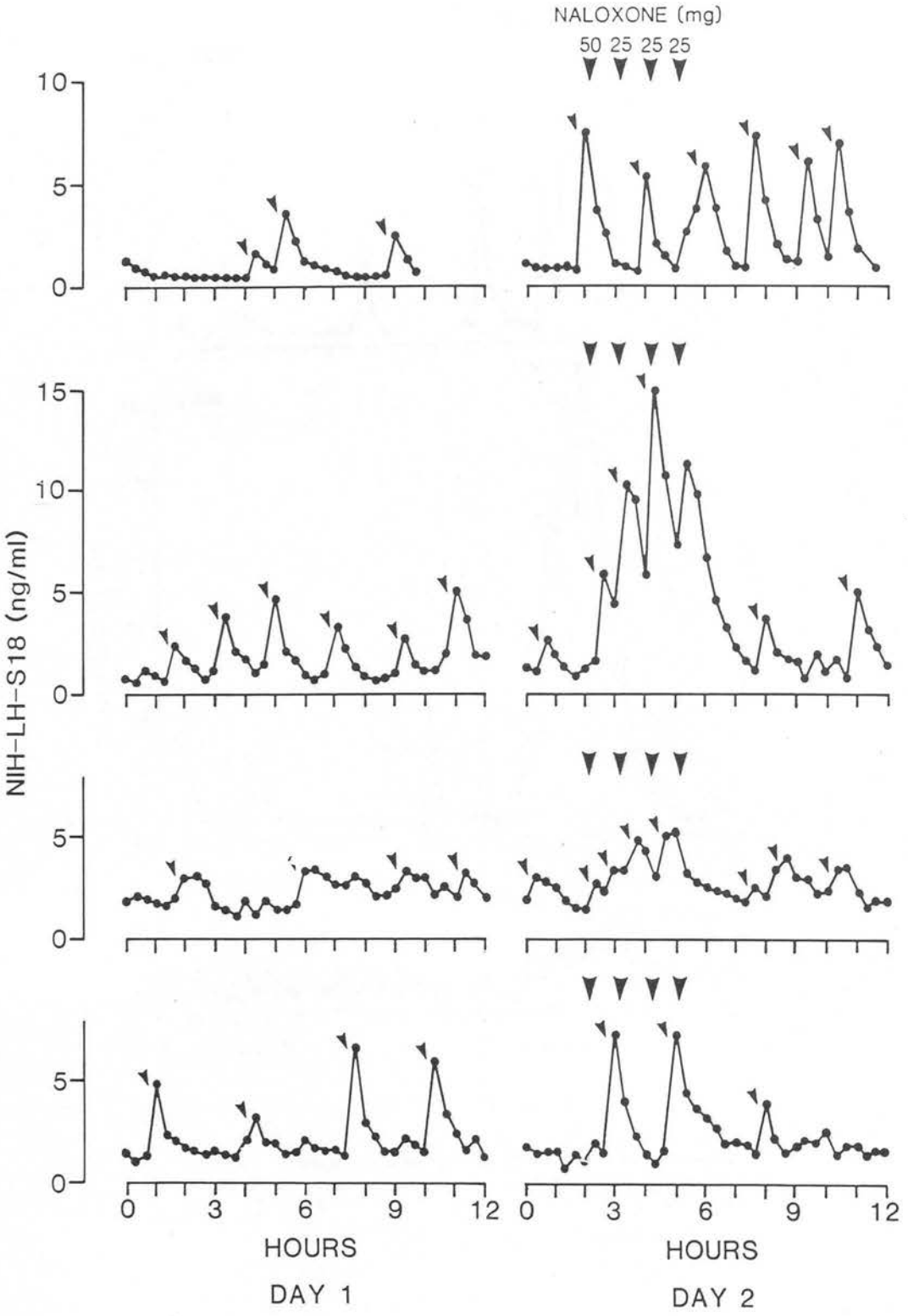

Fig. 3.5.14d

Experiment 5. Effects of naloxone on plasma LH concentrations at four times of year in a PINx Soay ram living out-of-doors. On each occasion, day 1 was a control period, and on day 2 a total dose of 125mg naloxone was given i.v. over 4 hours. ▼ indicates a significant LH pulse.

Table 3.5.15

Experiment 5: a comparison of the effects of naloxone and naltrexone on plasma LH prolactin levels in Soay rams in March and June.

	MEAN LH LEVEL ng/ml			LH PULSE FREQUENCY pulses/6h			LH PULSE AMPLITUDE ng/ml			MEAN PROLACTIN LEVEL ng/ml		
	DAY 1	DAY 2	Δ	DAY 1	DAY 2	Δ	DAY 1	DAY 2	Δ	DAY 1	DAY 2	Δ
MARCH												
NALOXONE (n=4) 125mg	1.72 ± 0.74	3.37 ± 1.52	+1.65 ± 0.84	1.40 ± 0.30	2.25 ± 0.48	+0.86 ± 0.37	4.26 ± 2.03	4.87 ± 2.38	+0.62 ± 0.89	15.12 ± 6.01	8.62 ± 4.47	-6.51 ± 2.13
NALTREXONE (n=4) 125mg	1.43 ± 0.24	3.32 ± 1.52	+1.90 ± 1.35	0.93 ± 0.18	2.50 ± 0.50	+1.57 ± 0.42	5.96 ± 2.49	5.34 ± 1.75	-0.62 ± 2.62	13.07 ± 1.88	9.63 ± 3.09	-3.44 ± 5.64
JUNE												
NALOXONE (n=4) 125mg	1.99 ± 0.07	8.56 ± 2.36	+6.57 ± 2.35	2.13 ± 0.43	3.25 ± 0.48	+1.13 ± 0.43	3.58 ± 0.52	8.27 ± 1.82	+4.69 ± 1.48	47.02 ± 15.58	24.53 ± 5.15	-22.49 ± 10.27
NALTREXONE (n=4) 125mg	2.19 ± 0.26	6.07 ± 3.53	+3.87 ± 3.61	1.50 ± 0.20	2.00 ± 0.41	+0.5 ± 0.71	5.11 ± 0.97	6.85 ± 1.20	+1.74 ± 1.79	45.75 ± 9.37	40.47 ± 7.69	-5.28 ± 7.69

Values are mean ± S.E.M.

Intact and SCGx/PINx rams were used.

followed by 5mg injections at hourly intervals. In September and December naloxone was given to eight rams, and diprenorphine to the others. In December only two rams were included in the diprenorphine group because of the poor health of the other two rams. Blood samples were collected at 20 minute intervals for 10 hours on day three in March, June and September to investigate any prolonged effects of the opiate antagonist treatments.

3.5.3 Results

Fig. 3.5.13 illustrates several aspects of the seasonal physiology of the intact and "non-pineal" (ie. SCGx or PINx) rams used in this study. Fig. 3.5.14 a-d show plasma LH concentrations on the control and treatment days at the four times of the year in four individual rams. Fig 3.5.14 a and b depict intact rams, and suggest that greater responses to opiate antagonists occured in September and December than earlier in the year. Figs. 3.5.14 c and d depict two typical non-pineal rams. These rams appeared to show good responses to opiate antagonists in March, June and September, but no response in December.

Table 3.5.15 compares the effects of naloxone and naltrexone on LH and prolactin secretion in March and June. The day 2 treatment period was defined as being from the sample after the first injection of opiate antagonist to three hours after the last injection. Both treatments increased LH pulse frequency and mean LH levels and decreased prolactin levels relative to the control day values, however there were no significant differences between the effects of naloxone and the effects of naltrexone. In all subsequent analyses therefore the naltrexone treated animals have been combined with the naloxone

Table 3.5.16

Experiment 5: Effects of Naloxone on LH and Prolactin secretion in intact Soay rams (n=3).

TIME OF YEAR	OVERALL LH LEVEL ng/ml		LH PULSE FREQUENCY pulses/6h		LH PULSE AMPLITUDE ng/ml		MEAN PROLACTIN LEVEL ng/ml	
	CONTROL	NALOX	CONTROL	NALOX	CONTROL	NALOX	CONTROL	NALOX
MARCH	1.00 ± 0.17	1.95 ± 0.32*	0.62 ± 0.16	2.00 ± 0.58**	5.25 ± 3.29	3.58 ± 1.09	10.2 ± 1.89	4.74 ± 1.44*
JUNE	2.10 ± 0.30	2.34 ± 0.30	1.67 ± 0.33	1.67 ± 0.33	4.45 ± 1.19	5.38 ± 0.49	62.3 ± 18.0	39.9 ± 3.78
SEPTEMBER	2.71 ± 0.06	4.96 ± 1.05**	2.33 ± 0.33	3.66 ± 0.33**	2.19 ± 0.60	3.32 ± 0.89	3.67 ± 0.98	6.07 ± 1.09*
DECEMBER	2.44 ± 0.18	5.08 ± 0.31**	1.33 ± 0.17	3.66 ± 0.33***	3.72 ± 1.46	3.92 ± 1.11	1.73 ± 0.53	1.06 ± 0.48

Values are mean ± S.E.M.
Asterisks indicate significant differences from the respective control period: * $p<0.05$ ** $p<0.01$ *** $p<0.001$.
See text for explanation of statistics.

Table 3.5.17

Experiment 5: Effects of naloxone on LH and prolactin secretion in non-pineal Soay rams (n=5).

TIME OF YEAR	OVERALL LH LEVEL ng/ml		LH PULSE FREQUENCY pulses/6h		LH PULSE AMPLITUDE ng/ml		MEAN PROLACTIN LEVEL ng/ml	
	CONTROL	NALOX.	CONTROL	NALOX.	CONTROL	NALOX.	CONTROL	NALOX.
MARCH	1.92 ± 0.54	4.19 ± 1.51*	1.49 ± 0.15	2.6 ± 0.4**	5.02 ± 1.8	6.02 ± 2.09	16.5 ± 4.23	11.8 ± 3.55
JUNE	2.09 ± 0.33	10.3 ± 2.34**	1.9 ± 0.37	3.2 ± 0.37***	4.28 ± 0.73	8.86 ± 1.36**	36.9 ± 6.12	36.0 ± 10.6
SEPTEMBER	2.24 ± 0.14	3.69 ± 0.30*	2.8 ± 0.22	3.8 ± 0.21***	1.16 ± 0.05	1.82 ± 0.18	5.87 ± 1.43	10.8 ± 3.17
DECEMBER	2.42 ± 0.31	2.86 ± 0.38	1.0 ± 0.27	1.4 ± 0.4	3.62 ± 1.3	4.1 ± 0.9	5.4 ± 1.79	2.66 ± 0.97

Values are mean ± S.E.M.
Asterisks indicate significant differences from the respective control period: * $p<0.05$ ** $p<0.01$ *** $p<0.001$.
See text for explanation of statistics.

Table 3.5.18

Summary of ANOVAR with repeated measures on two factors for each parameter in each group.

	PARAMETER	EFFECT OF NALOXONE TREATMENT			EFFECT OF SEASON TREATMENT			INTERACTION OF NALOXONE AND SEASON		
		F	df	signif.	F	df	signif.	F	df	signif.
INTACT	MEAN LH	22.0	1, 2	p<0.05	13.1	3, 6	p<0.005	4.19	3, 6	p<0.05
	LH PULSE FREQ.	38.0	1, 2	p<0.05	5.76	3, 6	p 0.05	9.33	3, 6	p<0.025
	LH PULSE AMPL.	0.05	1, 2	ns	0.58	3, 6	ns	0.32	3, 6	ns
	MEAN PRL	2.62	1, 2	ns	18.9	3, 6	p<0.005	1.80	3, 6	ns
NON-PINEAL	MEAN LH	14.8	1, 4	p<0.025	5.91	3, 12	p<0.025	7.45	3, 12	p<0.005
	LH PULSE FREQ.	282	1, 4	p<0.001	13.4	3, 12	p<0.001	1.48	3, 12	ns
	LH PULSE AMPL.	18.9	1, 4	p<0.025	4.35	3, 12	p<0.05	4.48	3, 12	p<0.05
	MEAN PRL	0.04	1, 4	ns	11.3	3, 12	p<0.01	1.03	3, 12	ns

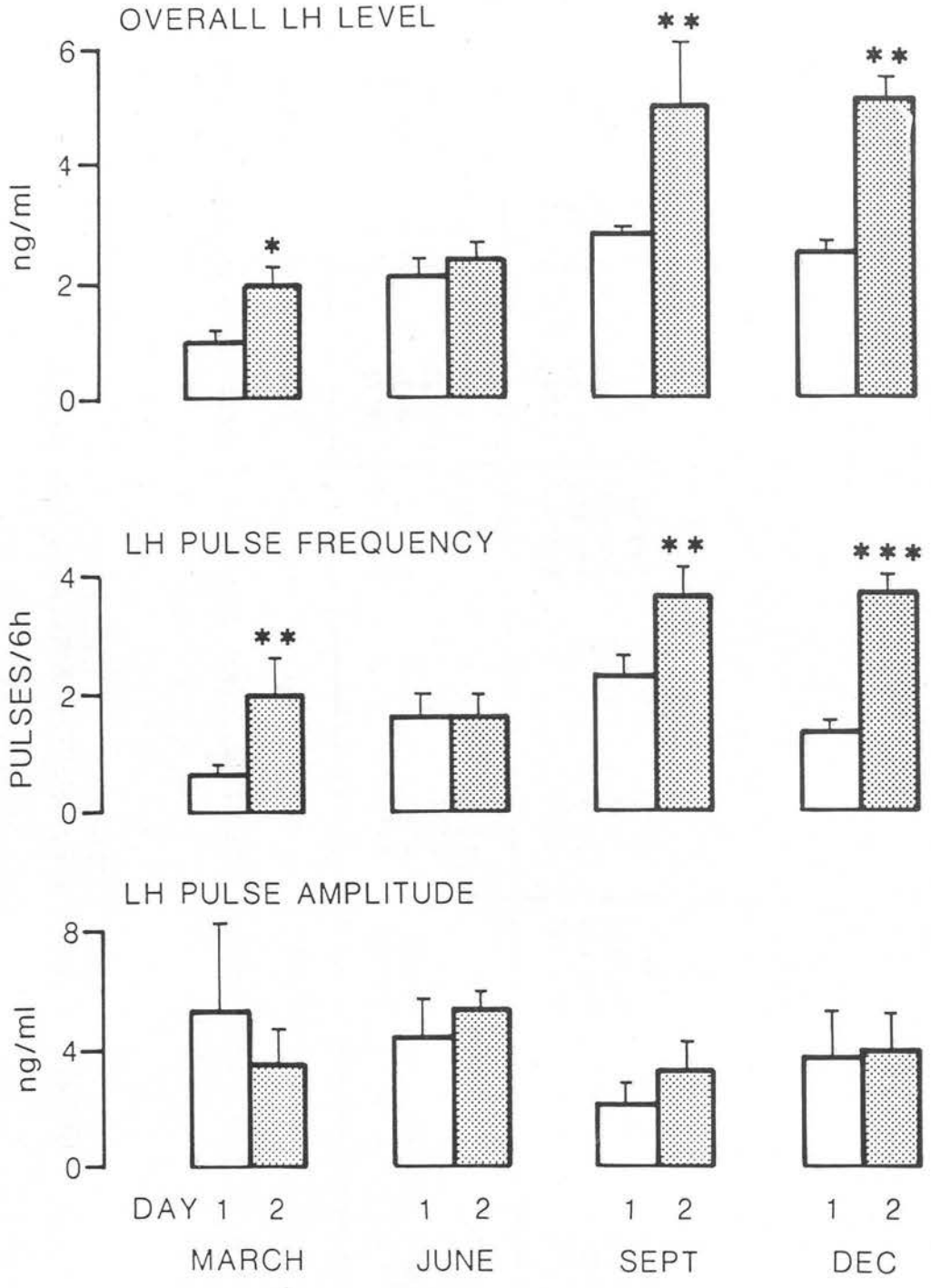

Fig. 3.5.19

Experiment 5. Summary of the effects of naloxone on LH secretion at four times of year in intact Soay rams. Values are mean ± S.E.M., n=3. Asterisks indicate significant effects of naloxone treatment : * $p<0.05$, ** $p<0.01$, *** $p<0.001$. See text for statistical methods.
In March and June the groups treated with naloxone and naltrexone have been combined.

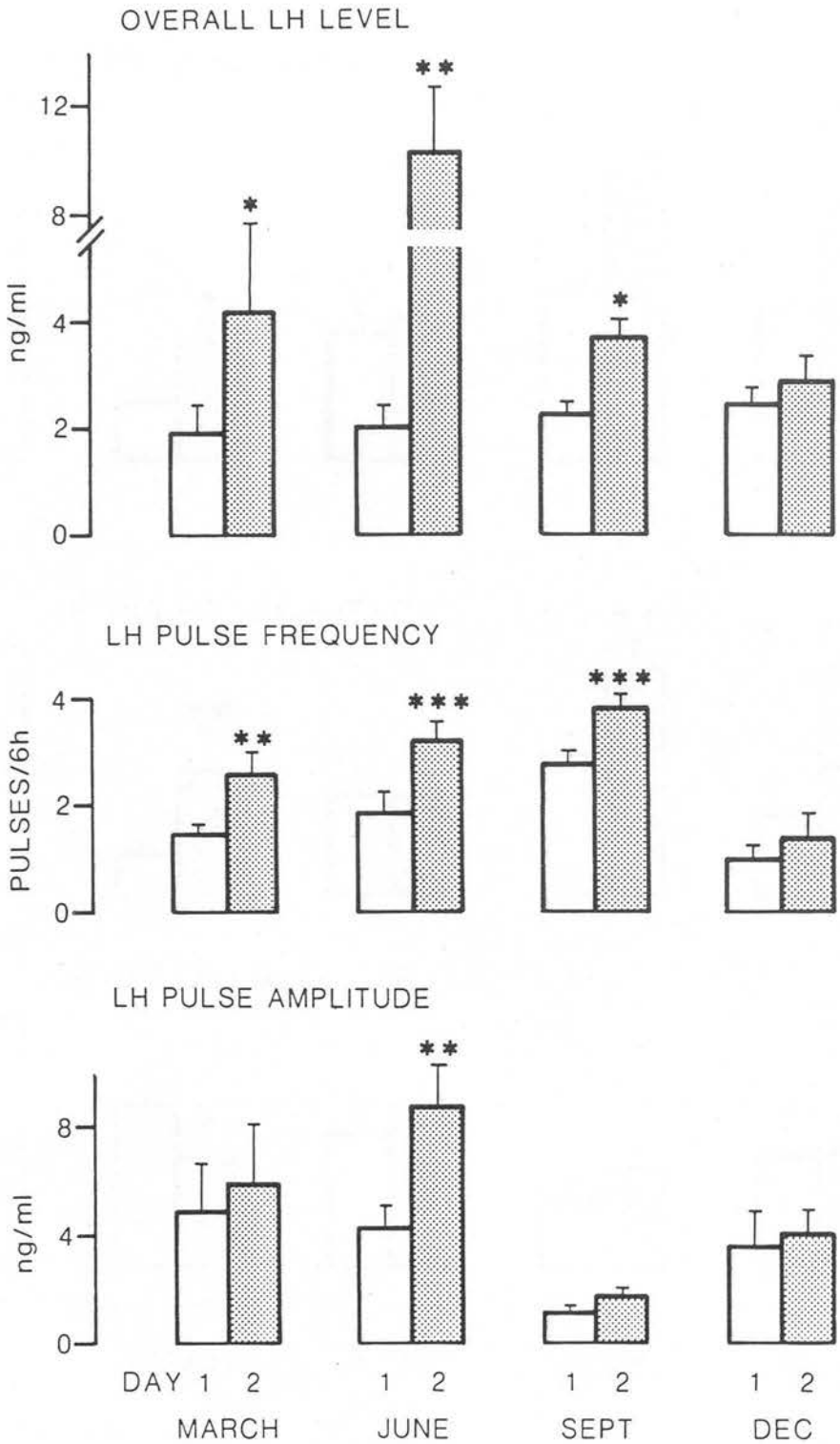

Fig. 3.5.20

Experiment 5. Summary of the effects of naloxone on LH secretion at four times of year in non-pineal Soay rams. Values are mean ± S.E.M., n=5. Asterisks indicate significant effects of naloxone treatment. : * $p<0.05$, ** $p<0.01$, *** $p<0.001$. See text for statistical methods.

In March and June the groups treated with naloxone and naltrexone have been combined.

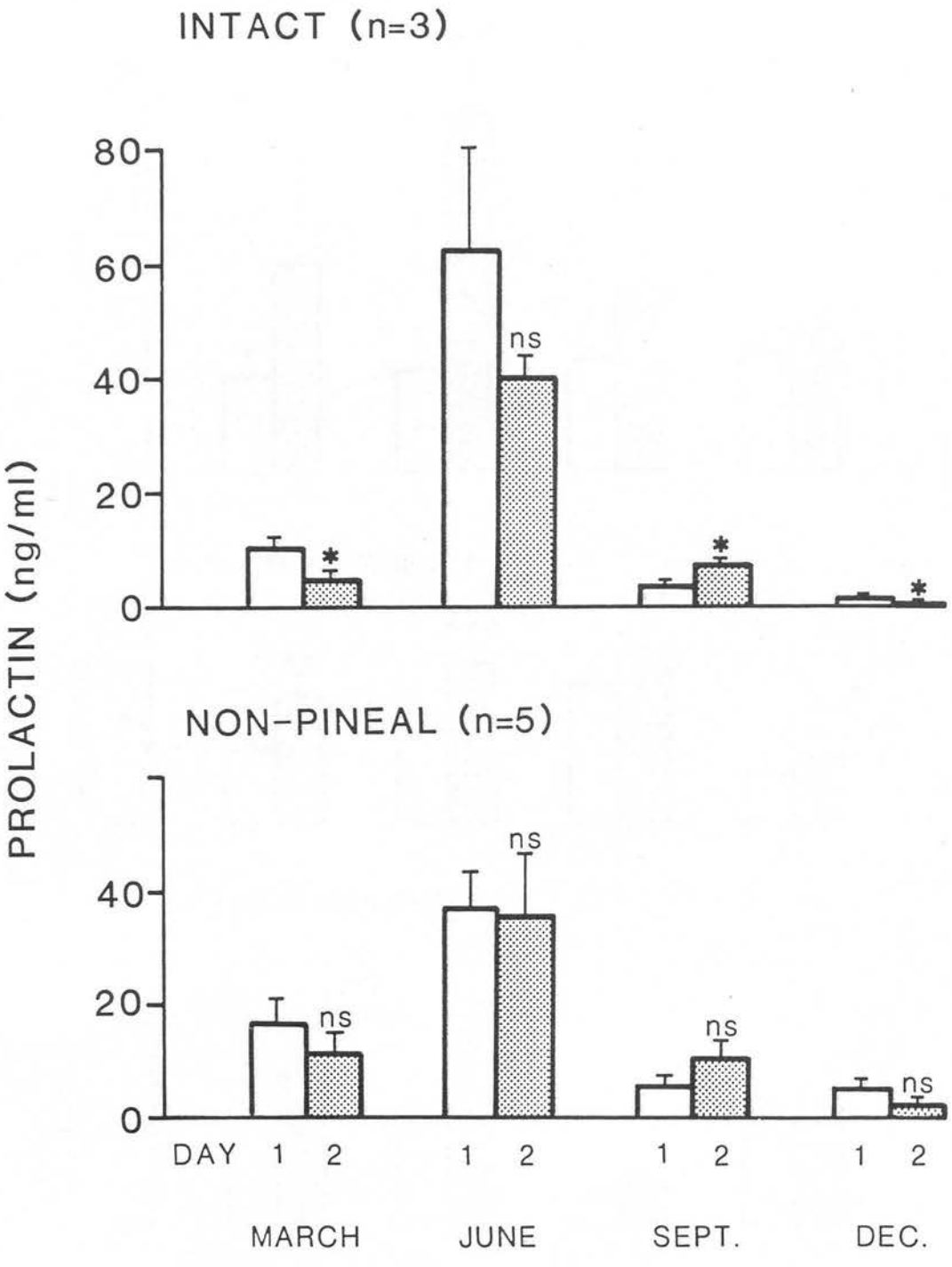

Fig. 3.5.21

Experiment 5. Summary of the effects of naloxone on plasma prolactin levels at four times of year in intact (n=3) and non-pineal (n=5) Soay rams. Values for each group are mean ± S.E.M. Asterisks indicate significant effects of naloxone treatment, * $p<0.05$.
In March and June the groups treated with naloxone and naltrexone have been combined.

treated animals; however the intact and the non-pineal rams have been analysed separately.

Tables 3.5.16 and 3.5.17 summarise the data for the four principle parameters measured in the intact and non-pineal rams respectively. The data were analysed by a series of two factor ANOVAR with repeated measures on both factors, ie. effect of naloxone and effect of season. Table 3.5.18 summarises the ANOVAR. In the intact rams there was a significant effect of season on mean plasma LH and prolactin levels and on LH pulse frequency as would be expected. There was a significant effect of naloxone on LH pulse frequency and hence mean LH levels. Interestingly, there was a significant interaction between the time of year (=effect of season) and the response to naloxone (=effect of naloxone) for both these parameters. To establish the time of year when naloxone induced the greatest response the simple effect of B (Naloxone treatment) at each level of C (time of year) has been calculated. Significance differences between control and treatment values at each time of year have been indicated by asterisks in table 3.5.16, and are summarised in fig. 3.5.19. There were no significant effects of either season or naloxone treatment on LH pulse amplitude. ANOVAR indicated no overall effect of naloxone on plasma prolactin levels, however the data suggest the possibility of a more complex response. The significant effects on prolactin level indicated by asterisks in table 3.5.16 were determined by paired t-tests. These indicated that opiate antagonists produced a significant inhibition of prolactin secretion in March and a significant stimulation in September, as summarised in fig. 3.5.21.

There were significant effects of season on all four parameters in the non-pineal group of rams, the effects on LH pulse frequency being

Table 3.5.22

Experiment 5: Summary of the effects of diprenorphine on LH secretion and plasma prolactin concentrations in intact and non-pineal Soay rams.

		OVERALL LH (ng/ml)		LH PULSE FREQUENCY (pulses/6h)		LH PULSE AMPLITUDE (ng/ml)		OVERALL PROLACTIN (ng/ml)	
		CONTROL	DIPREN.	CONTROL	DIPREN.	CONTROL	DIPREN.	CONTROL	DIPREN.
INTACT	MARCH (n=2)	1.88	0.90	1.55	0.50	7.37	1.45	4.95	2.56
	JUNE (n=2)	1.23	0.51	1.75	0.5	4.32	0.75	34.0	20.6
	SEPT (n=2)	2.54	1.68	2.0	2.0	1.57	1.27	0.88	2.01
	DEC (n=1)	2.71	1.78	2.5	1.0	1.6	0.9	0.78	1.09
NON-PINEAL	MARCH (n=2)	1.65	1.32	0.93	2.0	6.07	3.03	9.24	4.85
	JUNE (n=2)	0.99	1.94	2.0	2.5	1.66	2.41	34.4	25.3
	SEPT (n=2)	2.07	2.31	1.5	1.5	1.04	1.33	11.4	10.5
	DEC (n=1)	3.44	2.53	2.5	2.0	3.18	1.38	1.86	0.23
COMBINED	MARCH (n=4)	1.77 ± 0.16	1.11 ± 0.22	1.24 ± 0.25	1.25 ± 0.48	6.72 ± 2.79	2.50 ± 0.96	7.09 ± 2.18	3.70 ± 1.30
	JUNE (n=4)	1.11 ± 0.13	1.22 ± 0.64	1.88 ± 0.13	1.5 ± 0.03	2.99 ± 1.19	1.85 ± 0.58	34.3 ± 3.36	22.9 ± 1.51
	SEPT (n=4)	2.31 ± 0.20	2.0 ± 0.28	1.75 ± 0.25	1.75 ± 0.48	1.31 ± 0.27	1.30 ± 0.24	6.16 ± 3.36	6.24 ± 2.97
	DEC (n=2)	3.08	2.16	2.5	1.5	2.39	1.14	1.32	0.66

Values are mean ± S.E.M.

more pronounced than those on LH pulse amplitude. There was a very significant effect of naloxone treatment on LH pulse frequency, and there were also significant effects on pulse amplitude and mean levels. There was a significant interaction between effects of naloxone and time of year on mean LH levels and pulse amplitude, though no significant interaction was observed for the pulse frequency data. As with the data from intact rams, significant differences between pairs of means have been calculated as the simple effect of B (naloxone treatment) at each level of C (time of year). These have been indicated by asterisks in table 3.5.17, and are summarised in fig. 3.5.20. Naloxone treatment had no significant overall effect on plasma prolactin levels. however as with the intact animals there appeared to be a trend towards an inhibition of prolactin secretion in March and a stimulation in September (see fig 3.5.21).

Table 3.5.22 summarises the data from rams treated with diprenorphine. Clearly there was no stimulation of LH secretion; the data from the intact animals might suggest a marginal suppression of LH release. The effects of diprenorphine on prolactin levels resemble the effects of naloxone in that there is a trend towards suppression in March, but no effect or a stimulatory effect in September. To allow statistical analyses the intact and non-pineal animals were grouped together, ANOVAR with repeated measures on two factors was carried out on each parameter. Significant variance in mean LH and prolactin levels was observed due to the effect of season, but the diprenorphine treatment resulted in no significant differences in any of the parameters tested.

3.6 Experiment 6 A detailed study of the acute effects of naloxone on LH secretion.

3.6.1 Aims

The sampling interval adopted in experiment 5 was not always sufficiently frequent to permit an accurate measurement of pulse frequency. The data for individual rams illustrated in figures 3.5.14 a-d suggest that following naloxone treatment in September the pulse frequency calculated (tables 3.5.16 and 3.5.17) is a considerable underestimate. There were therefore two aims of this study;

1) To investigate in detail whether naloxone induced an increase in LH pulse frequency in sexually active rams.

2) To investigate whether the amplitude of LH pulses induced by naloxone correlated with pituitary responsiveness as determined by exogenous LHRH stimulation.

3.6.2 Materials and methods

Four 3½ year old Soay rams which had had their superior cervical ganglia removed shortly after birth and which were normally housed outside were used in this study. In late October the rams were temporarily housed indoors and blood samples collected initially at 15 minute intervals for 1½ hours, then at 4 minute intervals for 3½ hours, and finally at 15 minute intervals for 3 hours. 30 minutes into the 4 minute sampling period each ram was given a 100mg i.v. bolus of naloxone hydrochloride (Sterling-Winthrop, U.K.) dissolved in 5ml saline.

This "naloxone test" was followed by a "pituitary responsiveness test" three weeks later. The rams were cannulated again and housed indoors. After a short pre-bleed period, blood samples were collected at 4 minute intervals for 4½ hours. At the start of this sampling

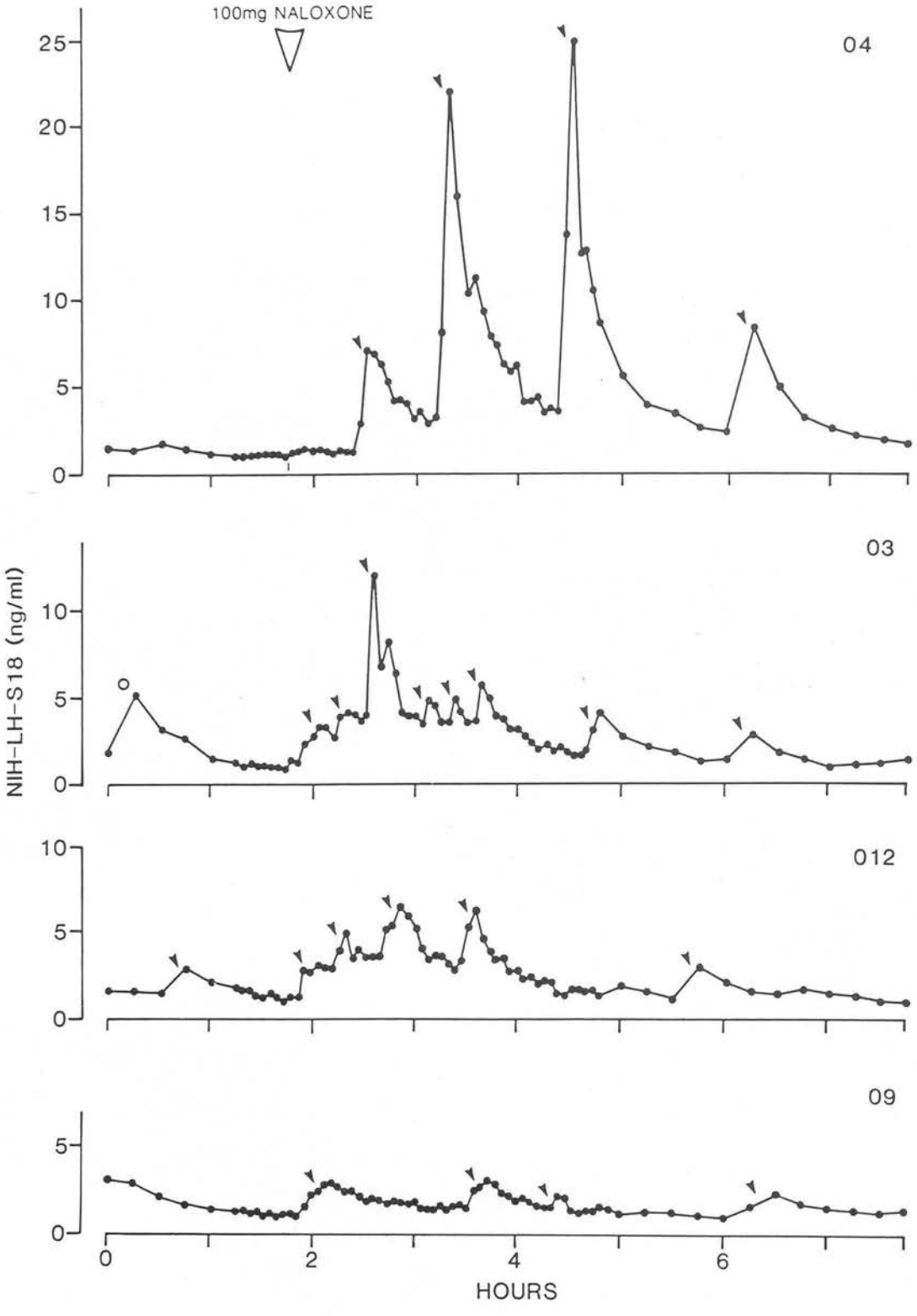

Fig. 3.6.23

Experiment 6. Effects of a 100mg i.v. bolus of naloxone on pulsatile LH secretion in four Soay rams in late October. ▼ indicates a significant LH pulse.

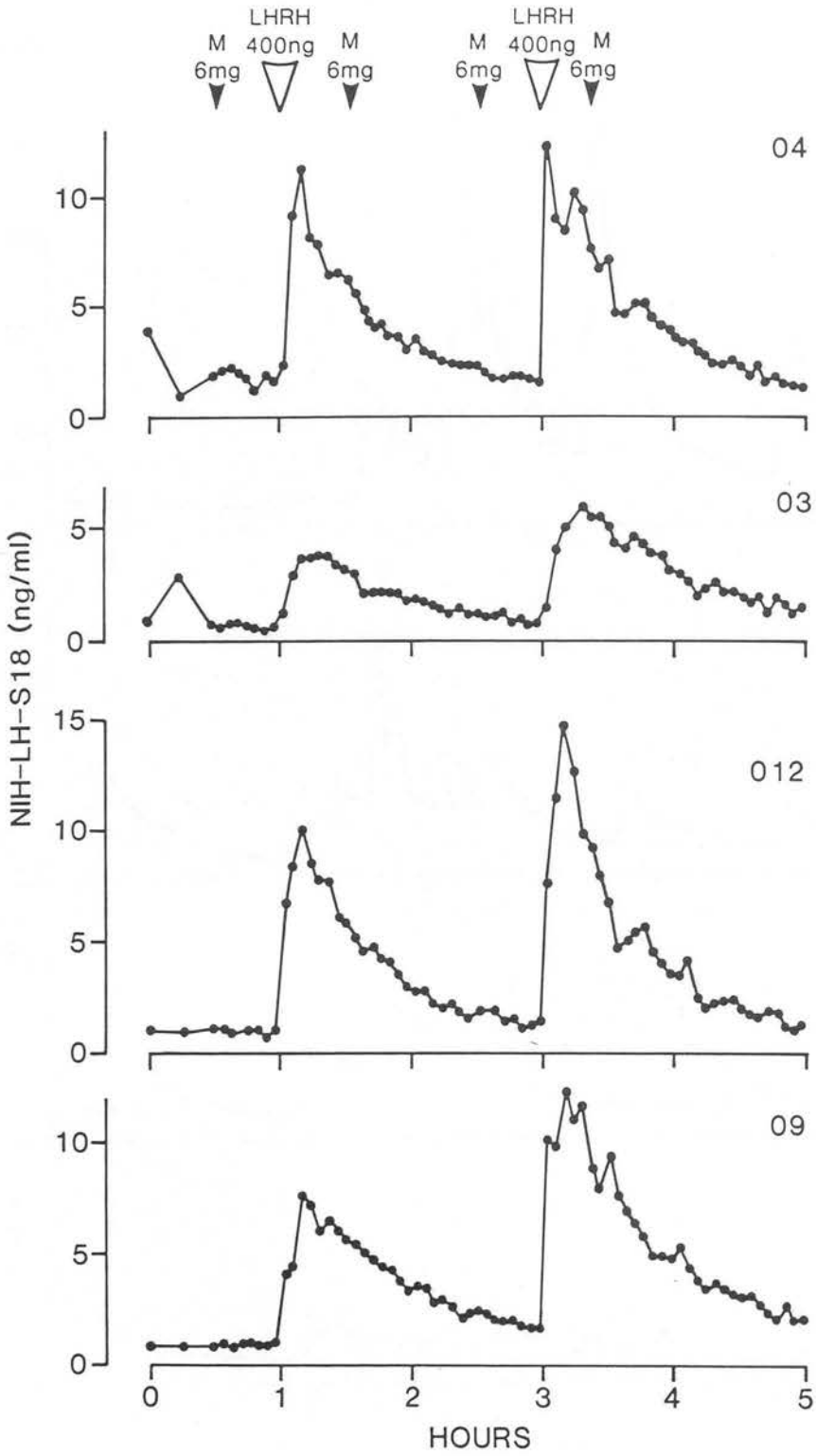

Fig. 3.6.24

Experiment 6. Effect of LHRH on plasma LH levels in four SCGx Soay rams in early November. Each ram received a total dose of 24mg morphine over 4 hours to suppress endogenous LHRH release. Two 400ng i.v. injections of LHRH were given two hours apart.

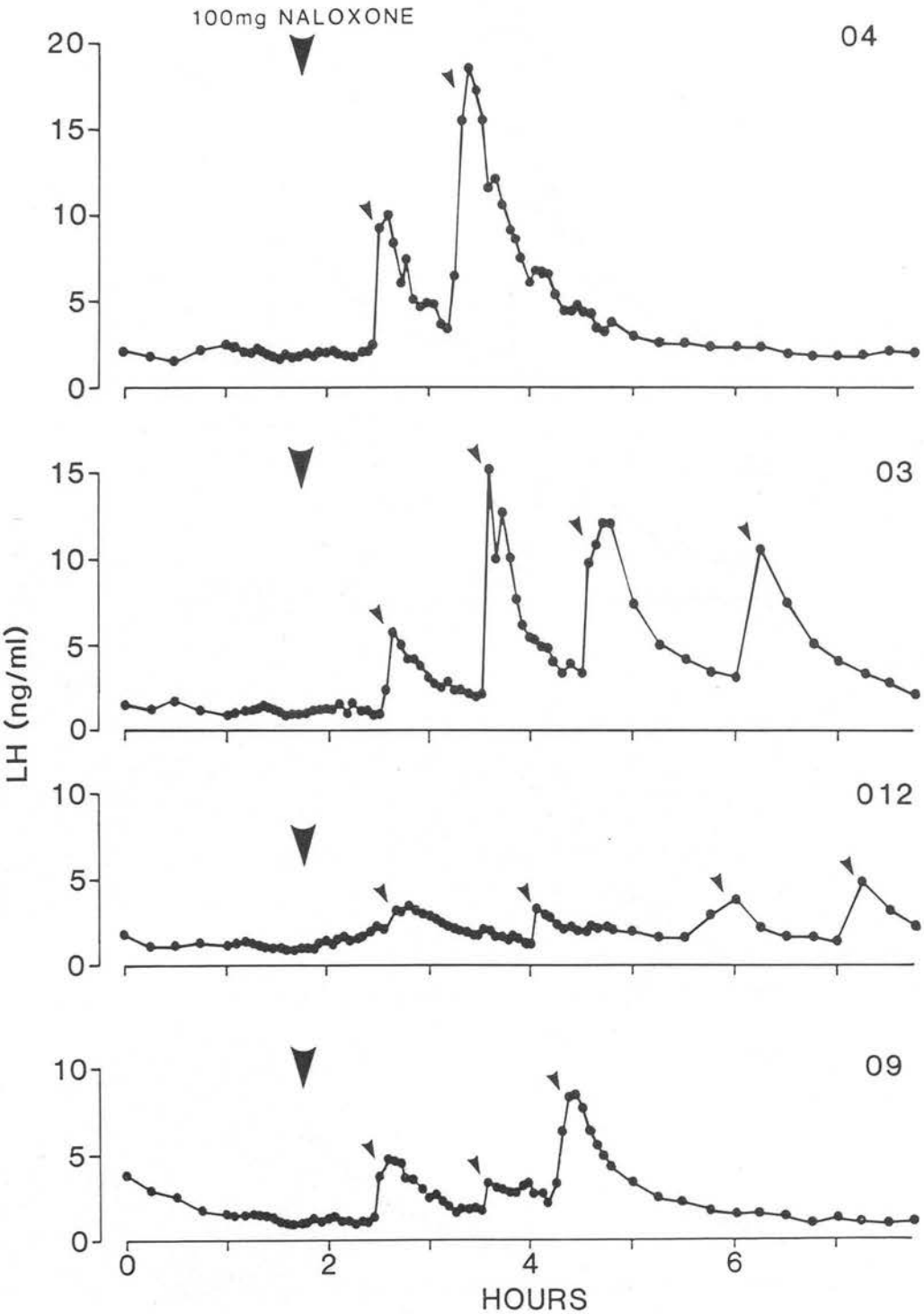

Fig. 3.6.25

Experiment 6. Effects of a 100mg i.v. bolus of naloxone on pulsatile LH secretion in four SCGx Soay rams in February. ▼ indicates a significant LH pulse.

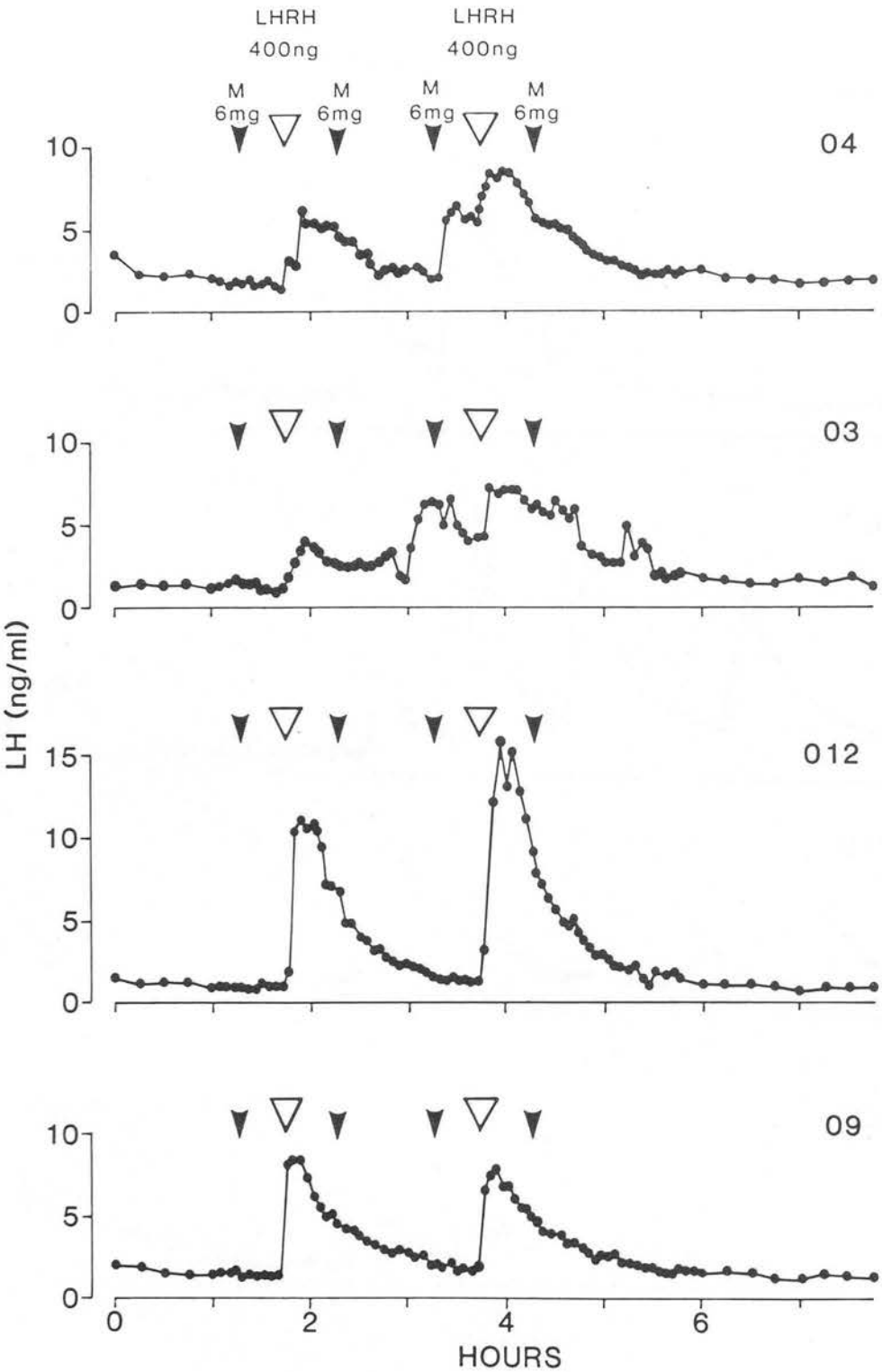

Fig. 3.6.26

Experiment 6. Effect of LHRH on plasma LH levels in four SCGx Soay rams in February. Each ram received a total dose of 24mg morphine over 4 hours to suppress endogenous LHRH release. Two 400ng i.v. injections of LHRH were given two hours apart.

Table 3.6.27

Experiment 6: Acute responses to 100mg Naloxone in October and February in four non-pineal rams.

PLASMA LH PARAMETERS	OCTOBER mean	OCTOBER range	FEBRUARY mean	FEBRUARY range
NALOXONE TEST (100mg)				
PULSE FREQUENCY (pulses/3h)	4.25 ± 0.95	3 - 7	2.5 ± 0.29	2 - 3
FIRST PULSE AMPLITUDE (ng/ml)	2.45 ± 1.09	1 - 5.7	4.68 ± 1.16	2.4 - 7.8
MAXIMUM PULSE AMPLITUDE (ng/ml)	8.08 ± 3.97	1.7 - 19.2	9.12 ± 2.97	2.4 - 15.1
MEAN PULSE AMPLITUDE (ng/ml)	5.0 ± 3.01	1.2 - 14.0	6.58 ± 2.18	2.2 - 11.5
TIME TO ONSET OF FIRST PULSE (minutes)	24 ± 7.7	10 - 46	44.5 ± 2.06	40 - 50
LHRH TEST (400ng x 2)				
AMPLITUDE OF RESPONSE TO 1st BOLUS (ng/ml)	7.13 ± 1.43	3.3 - 9.5	5.7 ± 1.46	3.0 - 9.3
AMPLITUDE OF RESPONSE TO 2nd BOLUS (ng/ml)	9.9 ± 1.70	5.2 - 13.3	8.05 ± 2.2	5.3 - 14.6
% INCREASE	44.0 ± 11	11 - 61	49.0 ± 22.4	-16 - 78
MEAN AMPLITUDE OF RESPONSE	8.52 ± 1.50	4.3 - 11.2	6.9 ± 1.75	4.2 - 12.0

Values are mean ± S.E.M., and range.

period each ram was given 6.25mg morphine sulphate i.v. dissolved in 3ml sterile 0.9% saline, and this dose was repeated at hourly intervals for three hours. 30 minutes into the 4 minute sampling period each ram was given an i.v. bolus of 400ng synthetic LHRH, dissolved in 3ml saline. This was repeated two hours later.

The "naloxone test" was repeated the following February using the same animals and experimental design, followed two weeks later with a second "pituitary responsiveness test" also of the same design as the autumn experiment.

3.6.3 Results

Figures 3.6.23-26 show the plasma LH concentrations from the individual rams in each of the four trials, and table 3.6.27 summarises pulse frequency and pulse amplitude characteristics following naloxone or LHRH treatment in October and February. On both occasions naloxone produced a substantial increase in pulse frequency. Mean pulse frequency following naloxone was 4.25 pulses/3h in October and 2.5 pulses/3h in February. Typical LH pulse frequency in untreated non-pineal rams in October is 1.5 pulses/3h, for example see control data for experiment 1, table 3.1.3, and less than 0.75 pulses/3h would be expected in February (see data for untreated non-pineal rams in experiment 5, table 3.5.17). In February there was a trend towards a greater LH pulse amplitude following naloxone treatment as compared to responses in October. Responses to LHRH however were similar in non-pineal rams at the two times of year, perhaps being marginally lower in February. The latency to onset of the first LH pulse following naloxone treatment was almost double in February.

3.7 Experiment 7 Effects of chronic opiate antagonist treatment on LH secretion

3.7.1 Aims

Several authors have proposed the hypothesis that EOP mechanisms mediate the negative feedback effects of gonadal steroids on hypothalamic LHRH release (Van Vugt et al.,1982; Bhanot and Wilkinson, 1983; Petraglia et al., 1984). In Soay rams, removal of gonadal steroid feedback by surgical castration results in very elevated pulsatile LH release after a few days (Lincoln and Short, 1980). The aim of this experiment was to investigate the above hypothesis by observing whether chronic treatment of rams with naloxone for a week would induce a functional castration response.

3.7.2 Materials and methods

This study was carried out using sixteen Soay rams of either three or four years age in April. Nine of the rams had been pinealectomized or ganglionectomized shortly after birth, and all the rams had been kept outside. The rams were housed inside in crates for the duration of the study but were maintained on a natural photoperiod. Deep dwelling jugular cannulae were inserted into all the rams prior to study, and left in situ for the duration of the experiment.

Eight of the rams (four intact, four non-pineal) recieved a 25mg i.v. injection of naloxone hydrochoride (Sterling-Winthrop, U.K.) every four hours for seven days. The other eight rams received injections of the saline vehicle only, and thus served as controls. Serial blood samples were collected at 20 minute intervals on three occasions. Firstly on day one of the experiment for eight hours prior to the start of naloxone treatment and for eight hours after (06.00 to 22.00 on day 1), secondly for eight hours on the third day of treatment (08.00 to 16.00

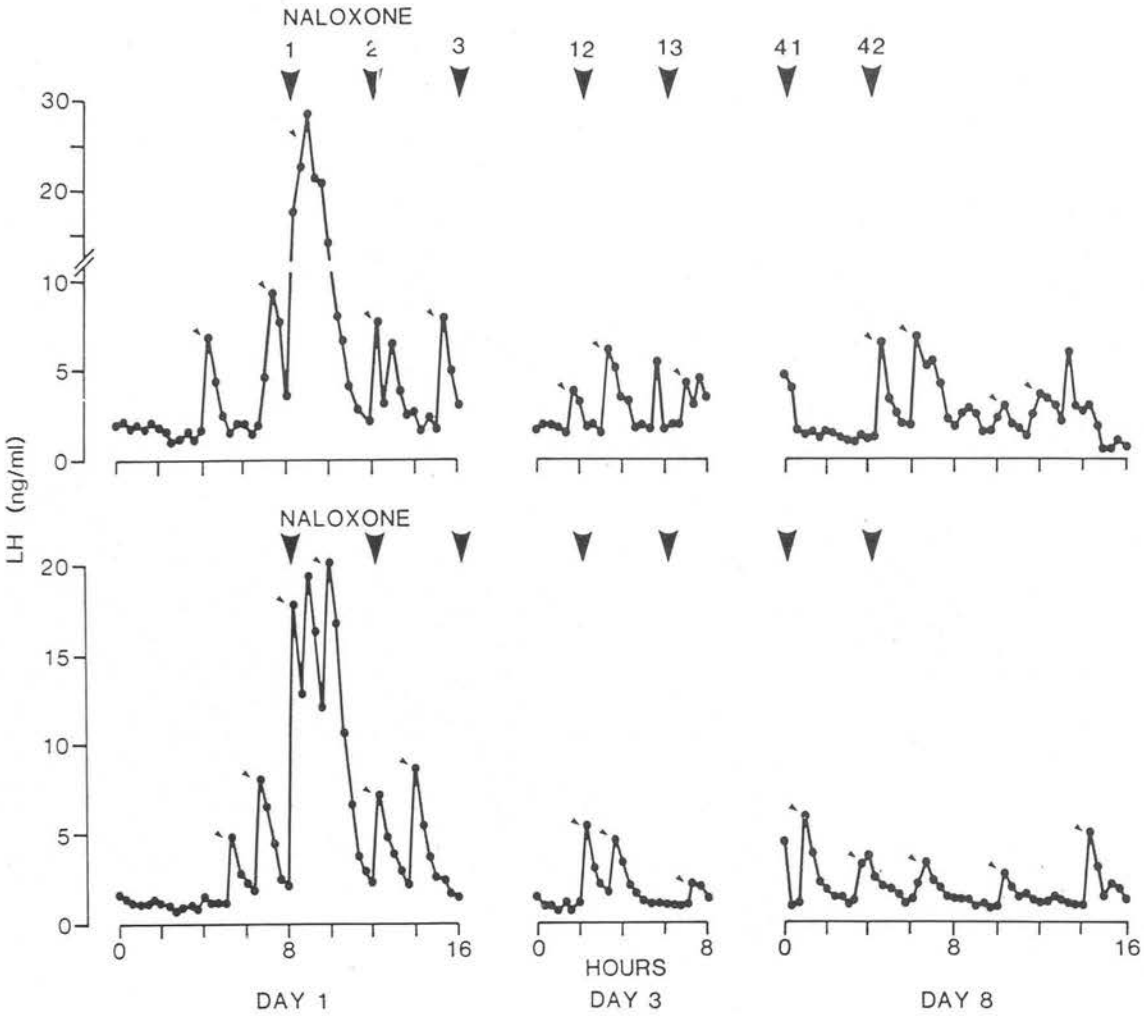

Fig. 3.7.28a

Experiment 7. Effect of chronic naloxone treatment on plasma LH levels in two Soay rams in April. Each ram received 25mg naloxone i.v. every 4 hours, starting after 8 hours sampling on day 1. Significant LH pulses are indicated by ▼.

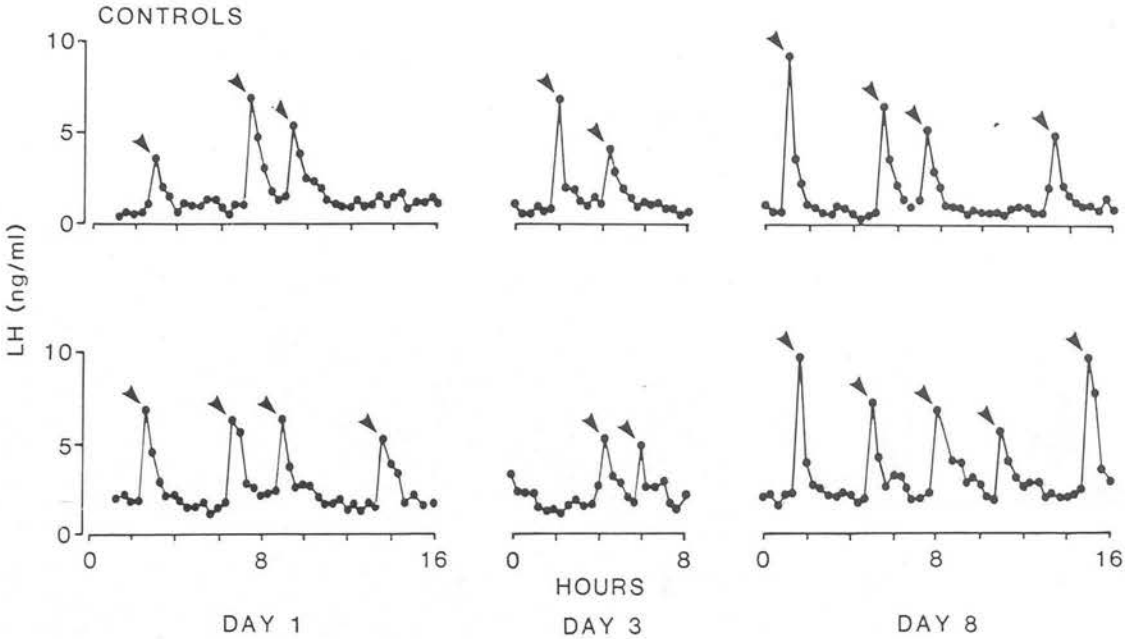

Fig. 3.7.28b

Experiment 7. Plasma LH levels in two control rams which received only the saline vehicle every 4 hours. Significant LH pulses are indicated by ▼.

Table 3.7.29

Experiment 7: Effect of chronic naloxone treatment on overall LH levels.

GROUP	DAY 1 PRE-TREATMENT 0 - 8h	DAY 1 NALOXONE 8 - 16h	DAY 3 NALOXONE 0 - 8h	DAY 8 NALOXONE 0 - 8h	DAY 8 POST-TREATMENT 8 - 16h
INTACT CONTROL (n=3)	1.90 ± 0.72	2.87 ± 0.60	1.26 ± 0.23	1.08 ± 0.13	1.53 ± 0.28
N.P. CONTROL (n=5)	2.33 ± 0.36	2.35 ± 0.39	2.34 ± 0.39	2.90 ± 0.50	2.65 ± 0.68
INTACT NALOXONE (n=4)	1.23 ± 0.33	3.19 ± 0.65	0.77 ± 0.30	2.06 ± 0.54	1.78 ± 0.85
N.P. NALOXONE (n=4)	2.48 ± 0.40	5.92 ± 1.32	2.24 ± 0.26	1.95 ± 0.31	1.53 ± 0.59
ALL CONTROL (n=8)	2.17 ± 0.33	2.54 ± 0.32	1.93 ± 0.32	2.22 ± 0.45	2.23 ± 0.47
ALL NALOXONE (n=8)	1.86 ± 0.34	4.55 ± 0.85[a]	1.51 ± 0.33	2.00 ± 0.29	1.66 ± 0.42

"a" indicates value differs significantly ($p<0.01$) from all other sampling periods for this group.
Values are ng/ml, mean ± S.E.M.

Table 3.7.30

Experiment 7: Effect of chronic naloxone treatment on LH pulse frequency.

GROUP	DAY 1 PRE-TREATMENT 0-8h	DAY 1 NALOXONE 8-16h	DAY 3 NALOXONE 0-8h	DAY 8 NALOXONE 0-8h	DAY 8 POST-TREATMENT 8-16h
INTACT CONTROL (n=3)	2.0 ± 0.0	2.66 ± 0.33	2.0 ± 0.0	1.66 ± 0.33	1.66 ± 0.33
N.P. CONTROL (n=5)	1.8 ± 0.2	2.0 ± 0.32	1.8 ± 0.2	2.2 ± 0.37	2.0 ± 0.32
INTACT NALOXONE (n=4)	1.75 ± 0.25	2.5 ± 0.29	1.5 ± 0.65	1.75 ± 0.63	0.5 ± 0.29
N.P. NALOXONE (n=4)	1.75 ± 0.25	3.25 ± 0.25	2.75 ± 0.25	2.25 ± 0.25	1.75 ± 0.25
ALL CONTROL (n=8)	1.88 ± 0.13	2.25 ± 0.25	1.88 ± 0.13	2.0 ± 0.27	1.88 ± 0.23
ALL NALOXONE (n=8)	1.75 ± 0.16	2.88 ± 0.23 [a]	2.13 ± 0.40	2.0 ± 0.33	1.13 ± 0.30

Values are pulses/8h, mean ± S.E.M.
"a" indicates value differs significantly ($p<0.05$) from pre-treatment sampling period and post-treatment sampling period.

Table 3.7.31

Experiment 7: Effect of chronic naloxone treatment on LH pulse amplitude.

GROUP	DAY 1 PRE-TREATMENT 0-8h	DAY 1 NALOXONE 8-16h	DAY 3 NALOXONE 0-8h	DAY 8 NALOXONE 0-8h	DAY 8 POST-TREATMENT 8-16h
INTACT CONTROL (n=3)	2.79 ± 0.67	3.62 ± 0.61	2.0 ± 0.29	1.46 ± 0.30	4.92 ± 0.93
N.P. CONTROL (n=5)	6.93 ± 2.58	4.82 ± 1.25	4.94 ± 1.86	6.23 ± 0.22	4.11 ± 0.92
INTACT NALOXONE (n=4)	2.75 ± 0.55	7.28 ± 2.45	1.50 ± 0.59	3.15 ± 1.02	1.74 ± 0.87
N.P. NALOXONE (n=4)	5.51 ± 0.37	7.16 ± 1.08	2.82 ± 0.12	3.48 ± 0.65	1.77 ± 0.33
ALL CONTROL (n=8)	5.34 ± 1.74	4.37 ± 0.80	3.84 ± 1.24	4.44 ± 0.89	4.42 ± 0.65
ALL NALOXONE (n=8)	4.08 ± 0.58	7.22 ± 1.24[a]	2.25 ± 0.30	3.34 ± 0.52	1.76 ± 0.31

Values are ng/ml, mean ± S.E.M.
"a" indicates value differs significantly ($p<0.01$) from all other sampling periods for this group.

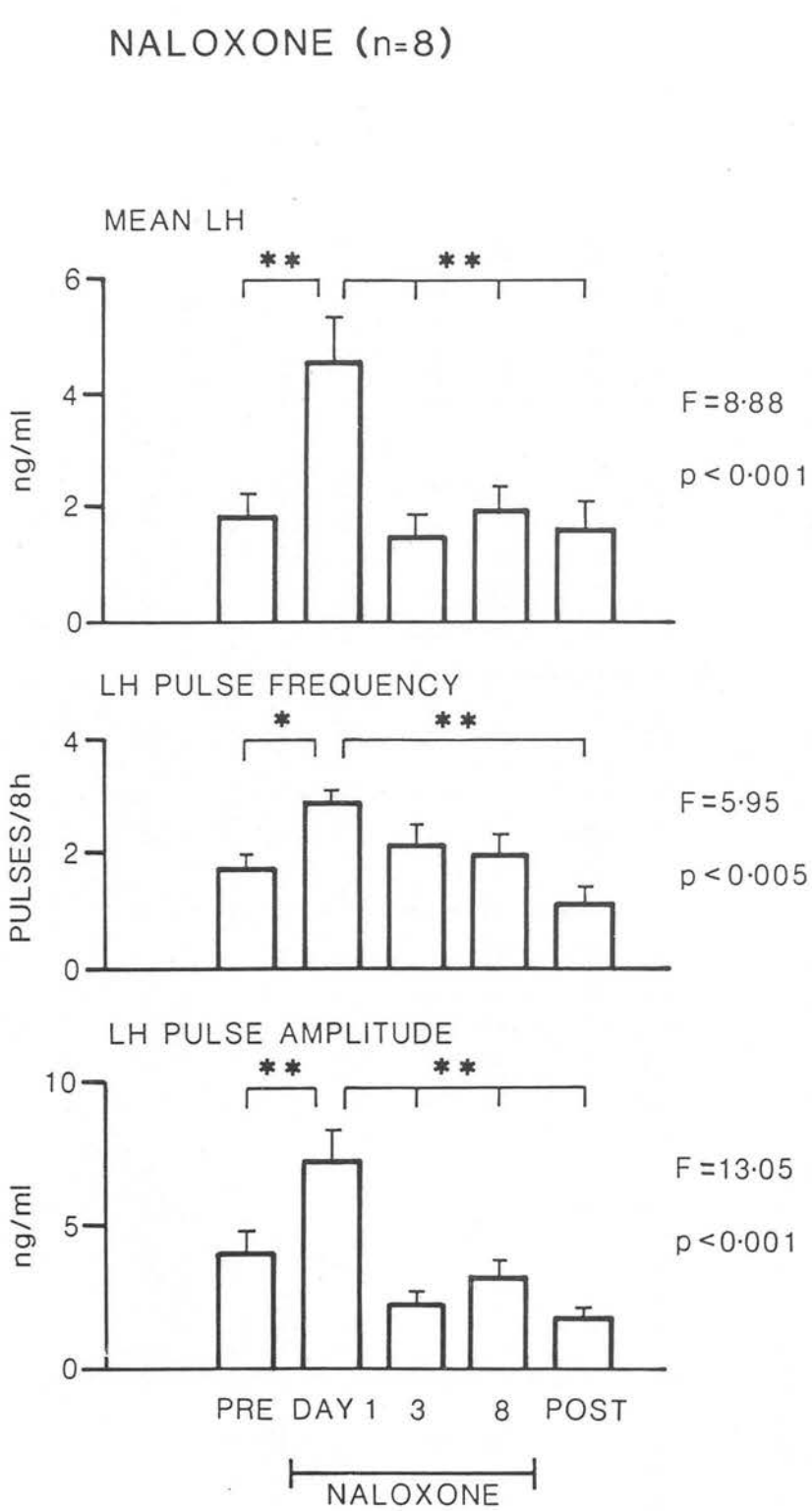

Fig. 3.7.32

Experiment 7. Summary of the effects of chronic naloxone treatment on LH secretion. Values are mean ± S.E.M., n=8. Significant differences are indicated by asterisks : ** p<0.01.

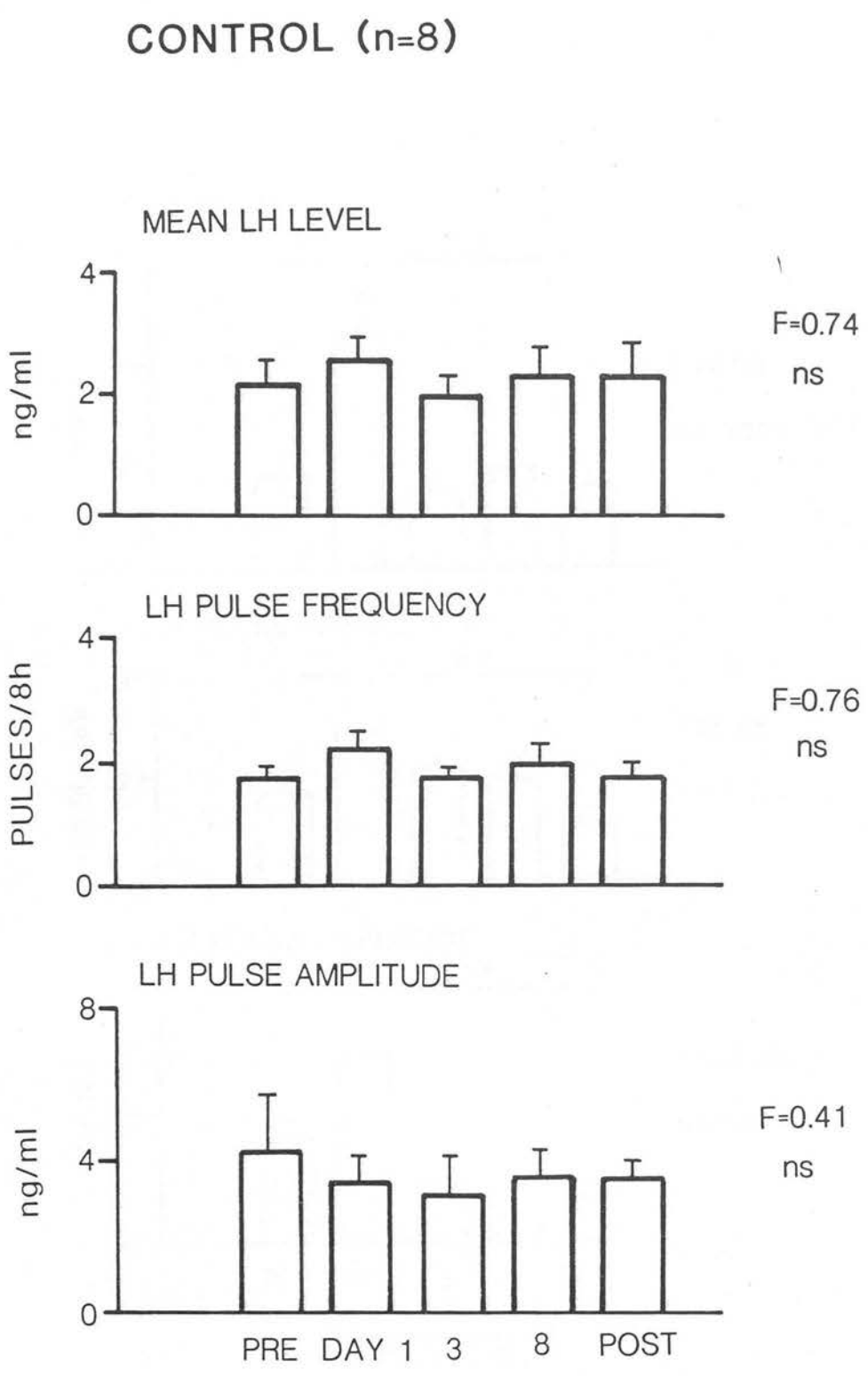

Fig. 3.7.33

Experiment 7. Summary of the effects of control treatment on LH secretion. Values are mean ± S.E.M.

on day 3) and finally for eight hours prior to the end of naloxone treatment and for eight hours post treatment on the last day of the experiment (06.00 to 22.00 on day 8).

3.7.3 Results

Fig. 3.7.28a shows plasma LH levels in two rams treated with naloxone, and fig. 3.7.28b shows two corresponding control rams. Tables 3.7.29,3.7.30 and 3.7.31 respectively summarise the mean LH levels, LH pulse frequency and LH pulse amplitude for each of the eight hour sampling blocks. For each parameter data from the control and naloxone treated group were analysed by one factor ANOVAR with repeated measures. In the control group there was no significant variance in any of the three parameters, whereas in the treatment group there were significant effects on mean LH levels ($F=8.88$, $p<0.001$), pulse frequency ($F=5.85$, $p<0.005$) and pulse amplitude ($F=13.15$, $p<0.001$). Where ANOVAR indicated significant effects, means for the eight hours sampling blocks were analysed using the Newman-Keuls' test to detect significant differences. These are indicated by subscripts in tables 3.3.29-3.3.31, and the results are summarised in figures 3.7.32 and 3.7.33. The naloxone treatment significantly elevated mean LH levels on the first day of treatment, this being attributable to increased pulse frequency and amplitude. There was no prolonged effect of naloxone treatment; all three parameters returning to pre-treatment levels by day 3, and also not differing significantly from the levels in the control rams.

3.8 Discussion

Experiment 1 clearly demonstrated that in sexually active intact and PINx rams morphine suppresses tonic LH secretion. This appears to be primarily a consequence of suppressed LH pulse frequency, though a decreased LH

pulse amplitude was also noted. The effects of morphine were antagonized by naloxone, which would suggest that morphine is exerting an effect via specific opiate receptors rather than acting as a non-specific CNS depressant. The dose of morphine used (approximately 0.5mg/kg b.w.) did not produce any noticable behavioural effects in this study. Although several studies have demonstrated that barbiturates and other anaesthetics will inhibit the oestrogen induced LH surge in ewes (Radford and Wallace, 1974; Dobson and Ward, 1977), there is less evidence for effects of non-specific CNS depressants on tonic LH secretion in sheep (Ellicott et al., 1975; Clarke and Doughton, 1983; Goodman and Meyer, 1984). This might also support the idea that the action of morphine is exerted via a specific mechanism, particularly since the last two studies also suggest that the effects of CNS depressants are related to the endocrine status of the sheep.

Experiment 1 also demonstrated that the naloxone plus morphine treatment actually stimulate LH pulse frequency and hence mean LH levels above that observed in the control period. One explanation is that the naloxone not only antagonised the suppressive effects of morphine but also blocked the inhibitory influence of endogenous opioid peptides (EOP) on LH secretion. An alternative explanation is that the morphine treatment actually enhanced the ability of naloxone to antagonise EOP suppression of LH. The pharmacology of opiate-receptor interactions is certainly complex (Smith and Loh, 1981). Several studies in the rat and mouse have demonstrated that acute or chronic treatment with both opiate agonists and antagonists increases the number of opiate binding sites in the brain as determined by subsequent in vitro binding of ^{3}H-naloxone or ^{3}H-dihydromorphine (Pert et al., 1973; Pert and Snyder, 1976; Lahti and Collins, 1978; Tempel et al.,

1982). This mechanism may account for the observations that naloxone pre-treatment enhances analgesic effects of morphine (Tang and Collins, 1978; Schulz et al., 1979). Since these opiate receptor responses may be very rapid (Pert and Snyder, 1976) it would seem possible that in certain EOP pathways morphine might actually potentiate the response to naloxone. Current studies in the lactating rat indicate that morphine pre-treatment lasting a week markedly enhances the stimulation by naloxone of oxytocin release (D.W. Lincoln pers. comm.).

The data in experiments 2 and 3 suggest that peripheral administration of β-endorphin does not alter LH or prolactin secretion at the doses used (approximately 5μg/kg b.w.). The responses to 0.25mg/kg b.w. morphine and morphine plus naloxone resembled those observed in experiment 1 using a higher dose of approximately 0.5mg/kg b.w. though the level of response was lower. The rams treated with β-endorphin in experiment 2 would therefore have been sensitive to the inhibitory effects of opiates. Although endocrine effects of peripherally administered β-endorphin have been demonstrated (rats: Rivier et al., 1977; Humans: Foley et al., 1979; Reid et al., 1981), using intracerebroventricular administration endocrine and behavioural responses have been observed using lower doses (Takahari et al., 1978; Foley et al., 1979; Kinoshita et al., 1980; Holaday and Loh, 1981; Motta and Martini, 1982). The half-life of β-endorphin injected peripherally is quite short. Catlin et al. (1981) reviewed nine separate studies of the plasma half-life of β-endorphin in the rat, the rabbit and man. The half-life of distribution is under 5 minutes, and the half-life of elimination in the circulation typically about 40 minutes, thus in experiment 2 the dose of β-endorphin used may be inadequate. Analogues of met-enkephalin which are resistant to degradation have

been demonstrated to be more potent in stimulating prolactin and growth hormone release and inhibiting LH release than met-enkephalin itself (Cusan et al., 1977; Von Graffenried et al., 1978; Stubbs et al., 1978), thus metabolic breakdown clearly affects the potency of opioid peptides injected peripherally. A second problem is that whereas alkaloid opiate agonists and antagonists rapidly cross the blood brain barrier, the penetration of β-endorphin into the central nervous system is very low (Houghten et al., 1980; Rapoport et al., 1980). Uptake of peripherally injected β-endorphin has been observed in the rabbit hypothalamus (Merin et al., 1980). Since the median eminence is outside the blood brain barrier it is possible that peripherally injected opioid peptides have neuromodulatory effects on the release of neurohormones from nerve terminals in the median eminence (Wilkes and Yen, 1981). If the action of peripherally given β-endorphin is only within the median eminence it is perhaps not suprising that no effects on LH pulse frequency were observed.

The doses of naloxone used in the pilot studies described in experiment 4 were not sufficient to significantly alter LH and prolactin secretion. The actual doses used (total doses 0.04 - 0.08mg/kg b.w.) were similar to those reported to suppress plasma prolactin and growth hormone levels and elevate LH levels in humans (Rubin et al., 1979; Morley et al., 1980; Ropert et al., 1981; Grossman et al., 1981; Lightman et al., 1981a). Considerably higher doses of opiate antagonists of 2 - 10mg/kg are needed to produce similar endocrine responses in rats (Blank et al., 1979, 1980; Ieiri et al., 1979, 1980; Van Vugt et al., 1981a,1982; Bhanot and Wilkinson, 1983). Very few studies have been carried out in sheep. Schanbacher (1982) reported in abstract form that 200mg naloxone per animal per hour

(=5mg/kg) were needed to significantly increase LH secretion, and recently Malven et al. (1984) demonstrated increased LH secretion in luteal phase ewes using a dose of 1.1mg/kg b.w.. One study reported that 2mg/kg was ineffective in raising LH levels in anoestrus ewes (Meyer and Goodman, 1983), but this lack of response may well be due to the seasonal condition of the ewes as will be discussed later.

On each occassion in experiment 5 a total dose of 125mg was given to all the rams over a period of 4 hours. This represents a dose of 4-6 mg/kg b.w.. Reports of the plasma half life of naloxone vary between 20 minutes and an hour (Berkowitz et al., 1975; Tallarida et al., 1978), thus it is not suprising that the duration of effect was only a few hours. For statistical analyses the period of treatment was defined as being from the first naloxone injection to three hours after the last injection. The initial trials compared naloxone with naltrexone, an opiate antagonist which has a longer duration of action when given orally, probably because the primary breakdown product in the liver (6-naltrexol) also has antagonist activity. Naloxone given orally is rapidly metabolised by the liver by conjugation to glucuronic acid, hence its activity when given orally is about 2% of that when given parenterally (Fujimoto, 1970; Berkowitz et al., 1975; Gold and Pottash, 1982). The current study suggested that in rams, intravenous injection of naltrexone produced identical effects to naloxone, both in terms of level of response (table 3.5.15), and in duration of response. In the March and June trials, serial blood samples were also collected on the day after treatment, but in both the naloxone-treated and naltrexone-treated rams pulsatile LH secretion was similar to that on the pre-treatment day.

The effects of the mixed antagonist-agonist diprenorphine were also studied. Diprenorphine is a potent antagonist of certain exogenous opiates and is used routinely to reverse the effects of etorphine anaesthesia. In the current study no significant effects of diprenorphine on LH secretion were observed. Diprenorphine was able to suppress prolactin secretion at the times of year when naloxone was able to do so, but not in September and December. This result is consistent with the study of Lien et al. (1979) which tested the effects of five different partial agonists on prolactin levels in the rat and observed very variable responses.

Experiment 5 provides strong evidence for the existence of endogenous opioid mechanisms which inhibit LH secretion in rams. The data also show that there is a marked seasonal pattern of responsiveness to naloxone which is altered in rams which have been rendered "non-photoperiodic" by functional or actual pinealectomy. The interesting implication of the seasonal change in naloxone response is that it implies a seasonal change in the level of EOP inhibition of LH release.

Analysis of the data suggests that the stimulatory effects of naloxone on LH release were primarily due to increased LH pulse frequency. The individual data shown in fig. 3.5.14 suggest that the 20 minute sampling interval does not allow an accurate assessment of LH pulse frequency following naloxone stimulation, particularly in September when all the rams were sexually active. The effects of naloxone on LH pulse frequency at this time of year may therefore have been underestimated. Experiment 6 confirms that the responses seen do represent changes in LH pulse frequency. This is an important observation because it would suggest that the seasonal changes in LH

secretion observed in response to naloxone represent different levels of EOP inhibition of pulsatile LHRH release from the hypothalamus. It seems unlikely that the seasonal changes in pituitary responsiveness which have previously been demonstrated in Soay rams (Lincoln, 1977) could account for the current results because if this were the case significant changes in pulse amplitude would be expected rather than changes in pulse frequency. The data from experiment 6 also suggest that the differences in pulse amplitude that were observed between animals are the result of differences in amount of LHRH released from the hypothalamus rather than differences in pituitary sensitivity since there was no clear correlation between response to naloxone and response to exogenous LHRH.

Table 3.5.17 shows that in some rams significant increases in LH pulse amplitude were observed in addition to increases in LH pulse frequency. The data from experiment 6 do not rule out the possibility that where naloxone increases LH pulse frequency to a level that is more than the pre-treatment levels a pituitary priming effect may occur. Enhanced LH amplitude responses to multiple exogenous LHRH injections have previously been observed in rams (Stelmasiak and Galloway, 1977; Lincoln, 1979b; Lincoln and Short, 1980) and may form part of the mechanism by which increased LHRH pulse frequency creates an LH surge in the ewe (Aiyer et al., 1974; Martin, 1984). This mechanism would explain the large increase in plasma LH level seen in the ram depicted in fig. 3.5.14c.

In the intact rams the pattern of responsiveness to naloxone correlated well with the reproductive status of the rams. Responses were greatest in September and December when testicular activity was greatest (See fig. 3.5.13), reduced in March by which time testicular

regression had occurred, and almost absent in June when spontaneous testicular growth was beginning. The data from the "non-pineal" rams show a similar correlation between reproductive activity and response to naloxone, though their seasonal cycles are different to those in intact rams. Superior cervical ganglionectomized rams kept indoors on an artificial photoperiod maintain a high level of reproductive activity. Cycles of testicular regression and recrudescence do occur, but these are considerably reduced in amplitude compared to intact rams, and not in synchrony with changes in photoperiod (Lincoln, 1979a; Barrell and Lapwood, 1979). The pinealectomized and ganglionectomized rams kept outdoors and used in the current study showed more pronounced cycles in reproductive and physiological activity, though testicular regression still only reached about half that observed in the control rams (see fig. 3.5.13). Testicular recrudescence occured prematurely compared to controls, and regression also occured prematurely. In the absence of photoperiodic cues it would appear that other environmental stimuli such as nutrituion, temperature and social stimuli can influence the reproductive cycle in "non-pineal" sheep (Bittman et al., 1983a; Lincoln and Forbes, 1984). Similar effects of ganglionectomy and pinealectomy on seasonal cycles have been observed in other species kept under natural conditions (see chapter 1). Good responses to naloxone were seen in the "non-pineal" rams in March, June and September. It is an interesting observation that responses were least in December, the time of year when the "non-pineal" rams are at their reproductive and metabolic nadir. The decline in body condition in late autumn in non-pineal rams is quite marked - perhaps because having become prematurely reproductively active they do not have the metabolic reserves to overcome the decrease in food quality and poor climate.

The original hypothesis was that an EOP inhibitory mechanism might relay inhibitory influence of long days on the reproductive axis, thus one would expect to see the greatest responses to naloxone during sexual quiescence and no difference in naloxone sensitivity in "non-pineal" rams.

The current data indicates that this is not the case, and suggest that EOP inhibition is greatest when the rams are sexually active. The results are more consistent with the hypothesis that EOP mediate steroid feedback.

Experiment 7 was therefore designed to test further the hypothesis that EOP mediate steroid feedback by investigating whether a longer period of naloxone treatment might result in "functional" castration. The results were surprising in that all the rams initially reponded to the naloxone treatment, but by day three LH secretion had returned to pre-treatment levels, and no further effects were observed on day eight of the naloxone treatment. Although this study does not provide a simple answer to the original question, it is an interesting observation that the rams appeared to become tolerant to the effects of an opiate antagonist in the same way that animals become tolerant to opiate agonists. Tolerance to opiate antagonists as indicated by the failure of naloxone to maintain elevated tonic LH secretion has previously been observed in male rats (Owens and Cicero, 1981), and in ovariectomized female rats whether implanted with oestrogen or not (Gabriel and Simpkins, 1983). A recent study has confirmed that this tolerance to naloxone is reflected in changes in LH pulse frequency: naloxone infusions initially induce an increase in pulse frequency but within a few hours this returns to or actually falls below pre-treatment levels (R.A. Steiner, pers. comm.).

The complex nature of opiate-receptor kinetics has already been briefly discussed in this chapter. An increase in opiate receptors induced by naloxone treatment could account for the observed tolerance since this might create increased EOP binding, thus compensating for the initial antagonist effects of naloxone. Several other models have been proposed to explain opiate tolerance and dependence which might equally well explain the current data (Smith and Loh, 1981).

An alternative explanation for the lack of effect of chronic naloxone treatment is that changes in receptor affinity have been induced. Several different opiate receptor subclasses have been pharmacologically characterised on the basis of agonist affinity and pharmacokinetics (Lord et al., 1977; Kosterlitz and Paterson, 1980; Cox, 1982; Herz, 1983). These may represent the mechanism by which different EOP have differential actions. It has recently been suggested that there is a degree of plasticity in the specificity of receptors, and that chronic pharmacological stimulation with naloxone which is a high affinity mu antagonist might induce a formation of other receptor subclasses for which naloxone has a lower affinity than the endogenous ligand (Rees, 1984). Circumstantial evidence for this hypothesis may come from the study of Gabriel and Simpkins (1983). Constant release naloxone implants were unable to enhance tonic LH secretion for more than one day post i plantation, however they were able to antagonise morphine induced analgesia and LH suppression for at least eight days. Since morphine is a mu receptor agonist it would appear that the tolerance to naloxone was due to endogenous opioid ligands acting on alternative subclasses of receptors.

The existence of different opiate receptors may also explain the differential effects of naloxone on prolactin secretion observed in experiment 5. In December, March and June, naloxone and diprenorphine were able to suppress prolactin secretion in the majority of animals, though the results are not consistently significant. Prolactin levels were also measured in serial samples collected on the day after treatment (data not shown). In most cases the post-treatment day levels were significantly higher than those on the pre-treatment day. This was probably attributable to the stress of confinement in crates rather than a residual effect of the opiate antagonist treatment. It is therefore quite likely that in certain animals on the treatment day (day two) there would be stress induced increases in plasma prolactin levels. The suppressive effects of opiate antagonists on prolactin secretion will therefore be masked to some extent, and the apparent increase in prolactin levels on the treatment day in September may reflect a seasonal decrease in the ability of naloxone to antagonise stress induced prolactin release. Slight effects of confinement stress on LH secretion have been reported (Rasmussen and Malven, 1983), however no stress effects were observed in the current experiments. Day three post-treatment LH secretion was similar to that on the pre-treatment day (data not shown), and no changes were observed in the control rams which were housed in crates for eight days in experiment 7 (see table 3.7.29-31).

There was clearly no simple correlation between the LH and prolactin responses to opiate antagonists in experiment 5. Diprenorphine partly suppressed prolactin levels but had a negligible effect on LH secretion. Lien et al. (1979) have previously observed that other partial agonists can suppress prolactin secretion without

altering other endocrine systems such as growth hormone release. On several occas ions naloxone markedly enhanced LH pulse frequency but did not affect prolactin secretion. Other studies have also demonstrated differential effects of naloxone on LH and prolactin secretion (Blank et al., 1980;Delitala et al., 1981; Grossman et al., 1982a; Rogol et al., 1984). It therefore seems possible that different opiate receptors and endogenous peptides are involved in the control of different neuroendocrine systems. The differential effects of naloxone may be a result of it having a higher affinity for the mu receptor than for other receptors (Rees, 1984). Highly specific antagonists for delta, kappa and sigma receptors have not been available, and preliminary studies using a variety of specific agonists for different receptor subclassess have not yet unequivocally demonstrated the actual receptors and ligands that are involved in the control of LH and prolactin secretion (Delitala et al., 1983b; Pfeiffer and Pfeiffer, 1983; Leadem and Kalra, 1983).

3.9 Summary

1) Morphine, an alkaloid opiate, reduces LH pulse frequency and to a lesser extent LH pulse amplitude in sexually active rams, and these effects can be antagonised by naloxone. This indicates that specific opiatergic mechanisms are involved in the control of LH release.

2) Naloxone alone increases pulsatile LH secretion, indicating endogenous opioid peptide (EOP) inhibition of tonic LH release in Soay rams.

3) The responses to naloxone in intact rams are greater in the breeding season than when the rams are sexually quiescent. A correlation between the response to naloxone and sexual activity is also seen in rams which have a premature and attenuated breeding cycle

as a result of surgical or functional pinealectomy.

4) These results suggest that EOP inhibition is greater when gonadal steroid secretion is high, and support the hypothesis that opiatergic mechanisms relay the negative feedback effects of steroids on LHRH release in the hypothalamus.

5) Chronic naloxone administration induces an initial increase in LH secretion, but this effect is lost by day three of treatment, indicating that tolerance develops to opiate antagonist treatment.

Chapter 4

Measurement of β-endorphin by RIA in sheep plasma and tissues.

4.1 Experiment 8 A study of diurnal patterns of plasma β-endorphin in Soay rams housed under long and short artificial photoperiods.

4.1.1 Aims

In many studies in the rat and man immunoreactive β-endorphin has been detected in plasma (see chapter 1.2.8). Levels are increased in certain pathological states of the adrenal axis and can be altered by manipulation of normal adrenal steroid levels , but few studies have been able to ascribe any physiological significance to resting state levels of β-EP in the plasma. The aim of the current experiment was to investigate whether immunoreactive β-EP was detectable in ovine plasma and whether any diurnal variation existed, and to observe whether any correlations existed between β-EP levels and the reproductive and metabolic status of the rams.

4.1.2 Materials and methods

This experiment was carried out using eight mature Soay rams which had previously been housed indoors under alternating periods of long days(16L:8D) and short days (8L:16D) for several years as part of a larger study (Lincoln and Ebling, 1985). Four of the rams had received a silastic implant containing 4g melatonin (Sigma) 38 weeks prior to the first sampling period in this experiment, the other four rams were untreated intact controls. Implants were inserted subcutaneously and a local anaesthetic (Lidocaine) was used. On two occasions jugular cannulae were inserted and 10ml blood samples were collected at hourly intervals for 24 hours starting at 09.00. A further large blood sample was collected at 09.00 on the second day for gel chromatography. All samples were treated as previously described in chapter 2.1.19. A dim red 15w light was used during the dark period to assist sample collection. The rams

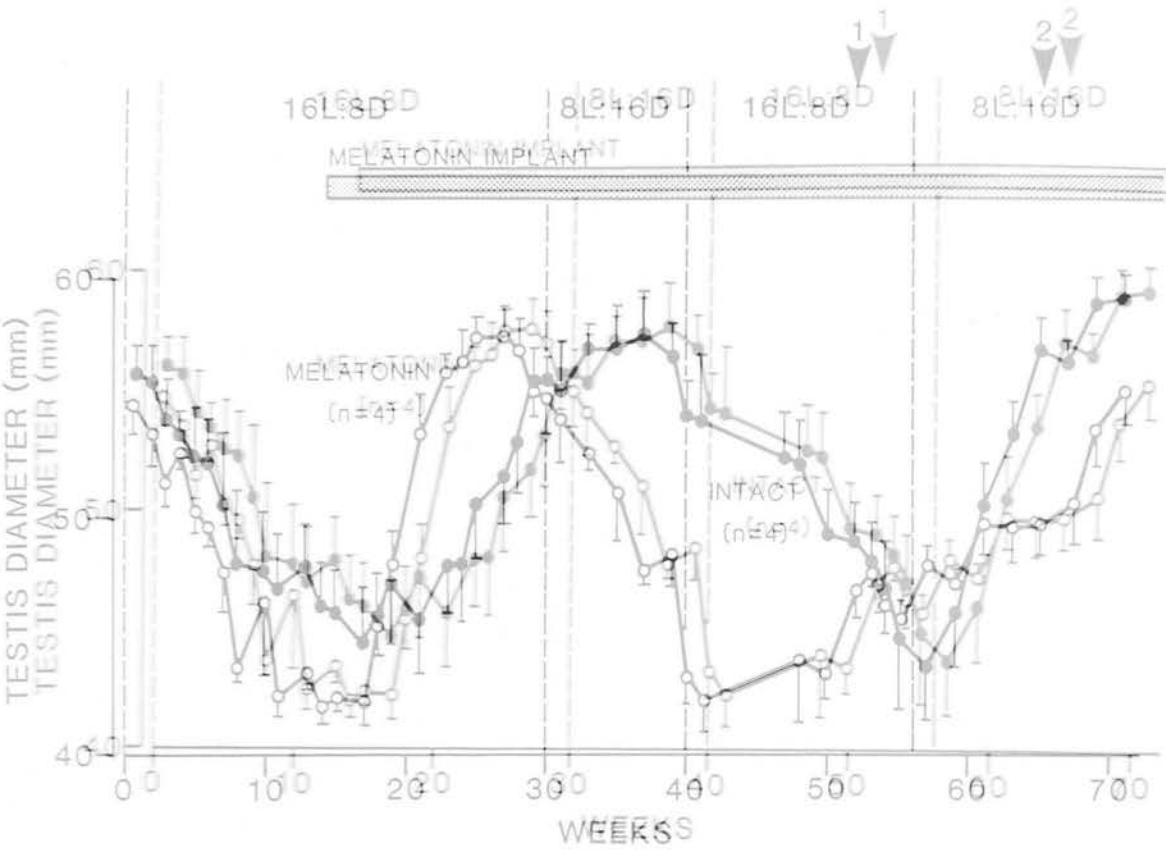

Fig. 4.4.3.1

Experiment 8. Photoperiodic and melatonin treatments and mean testicular diameter in Soay rams housed indoors. Values for testis diameter are mean ± S.E.M., n=4 for both groups. ▼▼ indicates the two occasions on which serial blood samples were collected for subsequent measurement of β-EP levels.

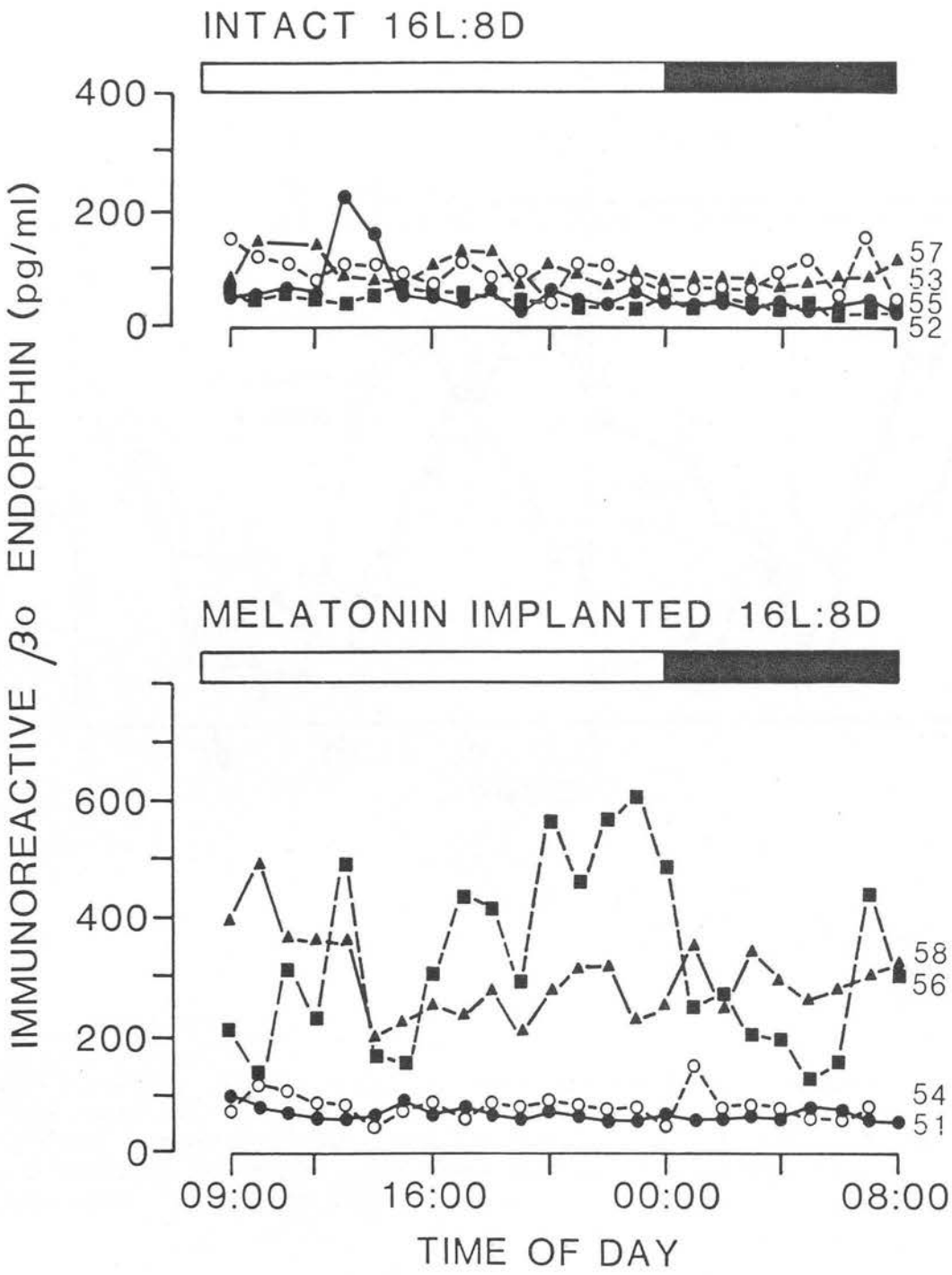

Fig. 4.1.2

Experiment 8. Individual hourly plasma β-EP levels in four intact rams (upper panel) and four melatonin-implanted rams (lower panel) sampled for 24 hours after twelve weeks exposure to 16L:8D.

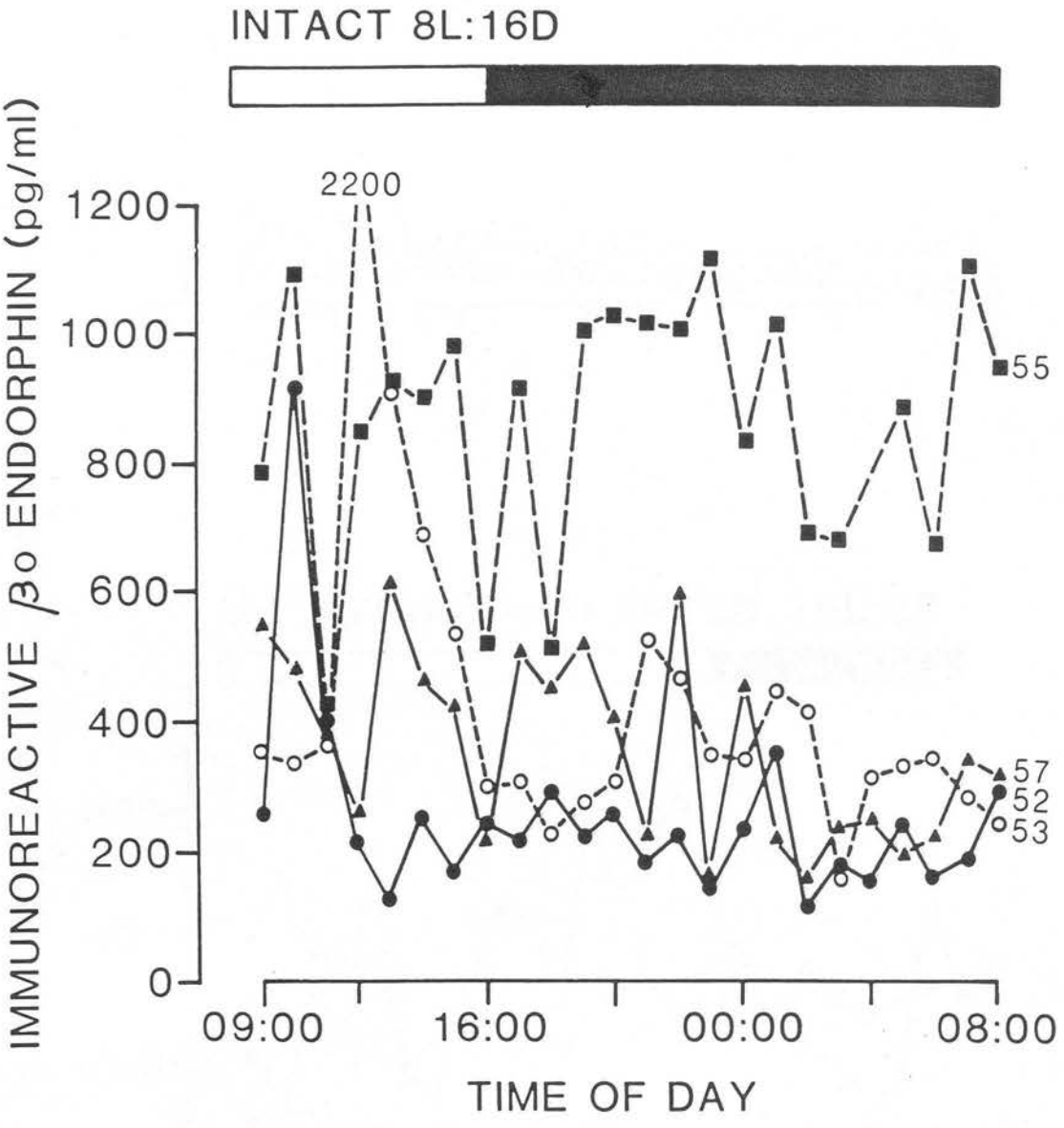

Fig. 4.1.3

Experiment 8. Individual hourly plasma β-EP levels in four intact rams sampled for 24 hours after nine weeks exposure to 8L:16D.

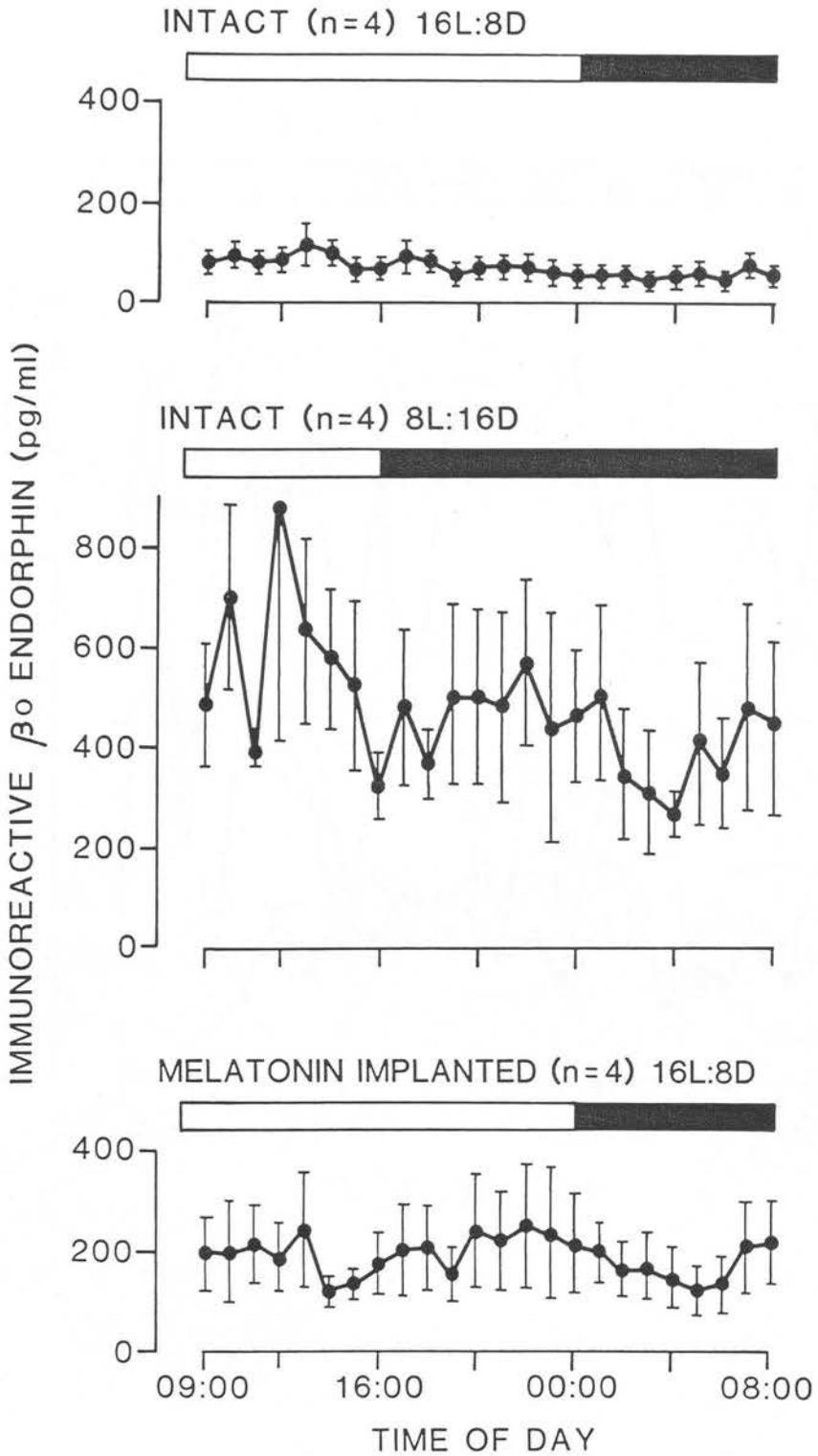

Fig. 4.1.4

Experiment 8. Comparison of hourly plasma β-EP levels in intact rams sampled under long and short days and in melatonin-implanted rams. Each point is the mean ± S.E.M., n=4.

were penned individually, and fed once daily between 08.00 and 09.00, water being freely available.

The first sampling period was twelve weeks after the rams had been transferred from short days (8L:16D) to long days (16L:8D). After a total of sixteen weeks under 16L:8D the lights were changed back to 8L:16D and the rams were blood sampled again after nine weeks exposure to this photoperiod.

Immunoreactive $\beta_o$endorphin was measured after VYCOR extraction by RIA using the B4.2 antiserum. Sephadex gel chromatography was carried out on selected samples as described in chapter 2.2, and the results are presented in section 4.4 in this chapter.

4.1.3 Results

Fig. 4.1.1 depicts the timing of the two sampling periods in relation to the photoperiod to which the rams were exposed, and illustrates the mean testis diameter for the two groups of rams. Fig. 4.1.2 shows the plasma immunoreactive $\beta_o$endorphin (β-EP) concentrations in the individual rams in the intact and melatonin implanted groups when sampled on long days, and fig. 4.1.3 shows the B-EP levels in the individual intact rams when sampled on short days. Fig. 4.1.4 shows the mean β-EP levels for each group.

No diurnal rhythmicity was evident in the individual β-EP concentrations. The mean values for each group were analysed by one factor ANOVAR with repeated measures. There was no significant effect of time of day on plasma β-EP levels in any of the three groups.

The data from the intact group strongly indicate that plasma β-EP levels are higher during exposure to short days than under long days. In the intact rams 24 hour overall mean β-EP levels were significantly higher on short days than on long days (paired 't' test: $p<0.01$).

The 24 hour mean β-EP concentrations in the melatonin implanted rams were not significantly higher than the intact rams when sampled under 16L:8D (Students 't' test), though the data in fig. 4.1.2 indicate that two of the melatonin implanted rams had plasma β-EP levels which more closely resembled the short day values in the intact group.

4.2 Experiment 9 A study of seasonal changes in plasma β-EP concentrations in Soay rams kept on artificial photoperiods.

4.2.1 Aims

The data in experiment 8 clearly demonstrate that plasma β-EP levels were higher under short days than under long days. The aim of this experiment was firstly to confirm that this indicated genuine seasonal variation rather than the effects of other factors during the sampling periods or assay differences, and secondly to investigate the relationship between plasma β-EP levels and photoperiod-influenced reproductive and somatic cycles.

4.2.2 Materials and methods

This experiment was carried out in intact adult Soay rams which had previously been housed indoors under artificial photoperiods for up to two years as part of the study described in chapter 5. The rams were individually penned and housed in two groups in separate light proof rooms. At the start of the study one group (group A) had been switched from short days to long days two weeks previously, and the other group (group B) had been maintained on short days for fourteen weeks. A blood sample was collected every week from each ram as described in chapter 2.1.19. This was taken between two and four hours after lights on. Food and water were both available ad libitum during the study. After eight weeks the photoperiods in the two groups were reversed, and

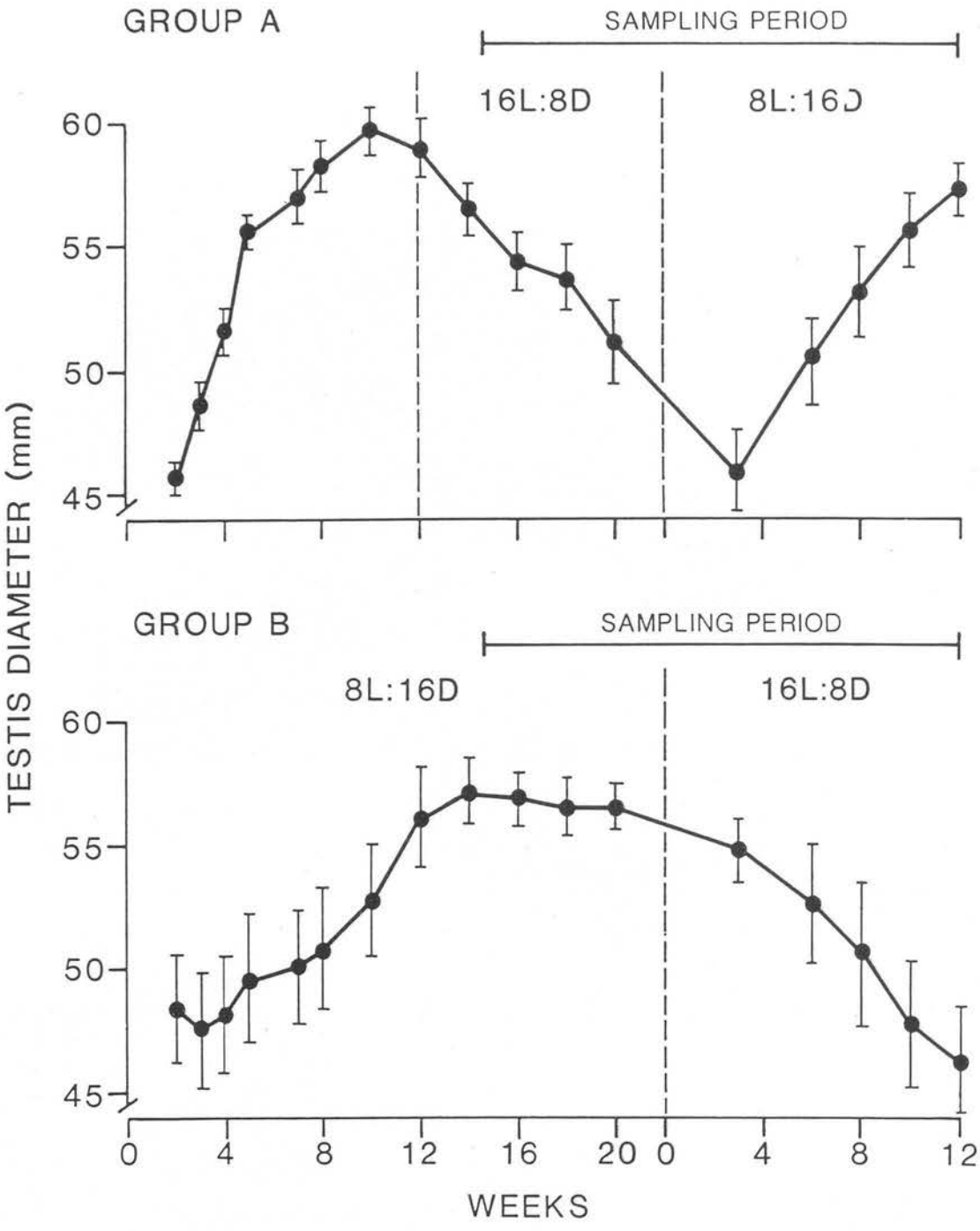

Fig. 4.2.5

Experiment 9. Mean testicular diameter in the two groups of Soay rams housed indoors under artificial photoperiod and blood-sampled weekly for measurement of β-EP levels. Each value is the mean ± S.E.M., n=6 for each group.

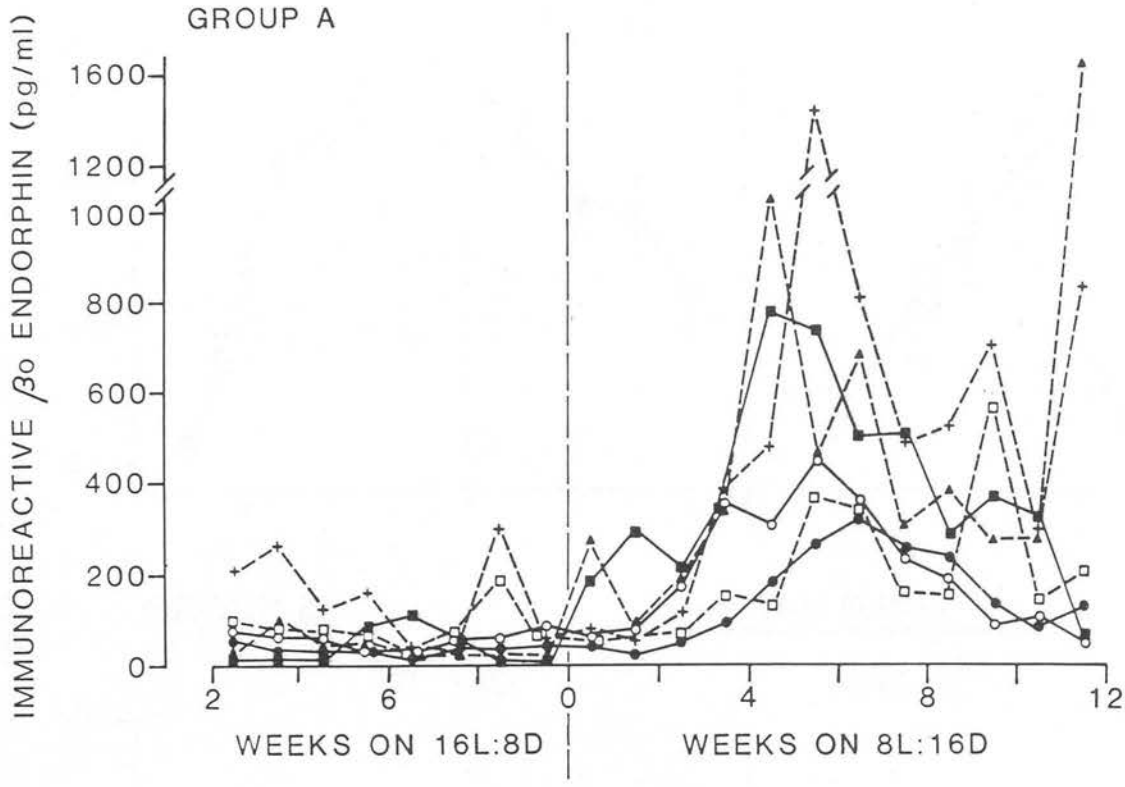

Fig. 4.2.6

Experiment 9. Individual weekly plasma β-EP levels in six Soay rams maintained on long days and subsequently transferred to short days (group A).

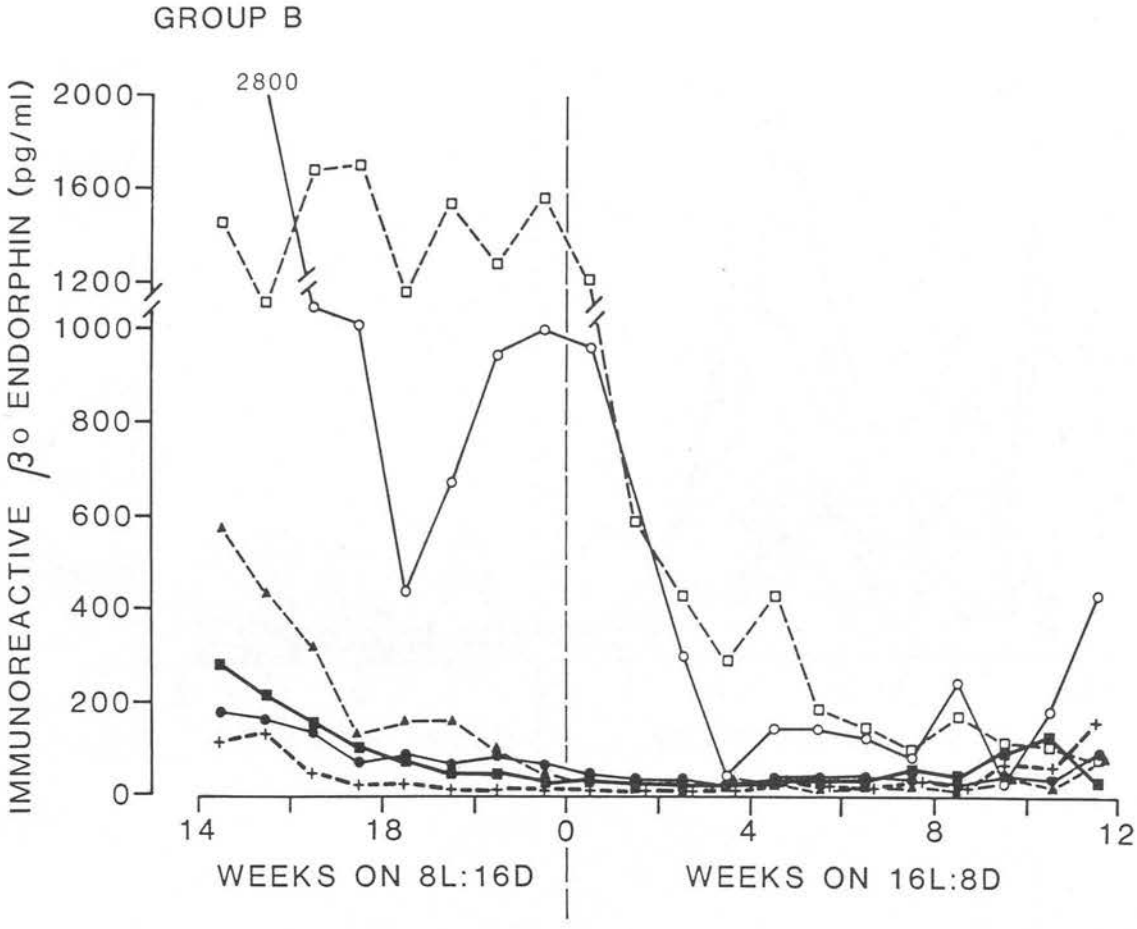

Fig. 4.2.7

Experiment 9. Individual weekly plasma β-EP levels in six Soay rams which had been maintained on short days and were subsequently transferred to long days (group B).

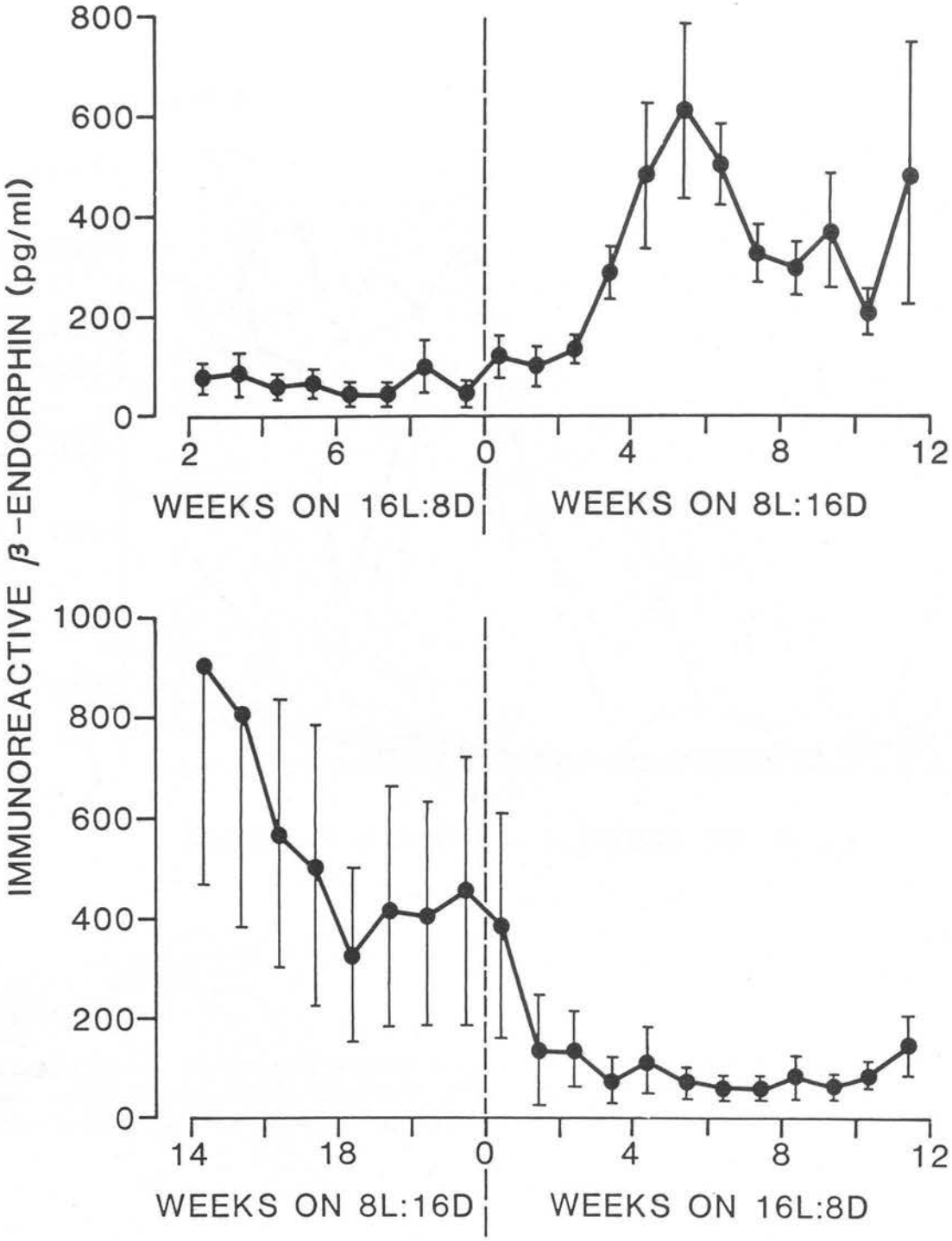

Fig. 4.2.8

Experiment 9. Mean plasma β-EP levels in the rams in group A (upper panel) and group B (lower panel). Each point is the mean ± S.E.M., n=6.

weekly sampling was continued for a further twelve weeks. An extra large plasma sample was also taken at either week eight or nine for gel chromatography. For the week prior to the final sample both groups of rams were exposed to skeleton photoperiods as described in chapter 5.2. Plasma β-EP levels were measured after VYCOR extraction by RIA using the 7.9.02 antiserum.

4.2.3 Results

Fig 4.2.5 depicts the mean testis diameter for the rams in each group in relation to photoperiod, and also indicates the sampling period. Figures 4.2.6 and 4.2.7 show the individual β-EP levels in the rams in group A and group B respectivly, and figure 4.2.8 shows the mean β-EP levels for each group. The mean data for each group were analysed by one factor ANOVAR with repeated measures, and both groups showed highly significant effects of time on β-EP levels (group A: $F=5.28$, $p<0.001$; group B: $F=2.87$, $p<0.001$). A Newman-Keuls' test indicated that in group A a significant increase in mean β-EP levels had occurred after four weeks exposure to short days compared to typical long day mean levels. The lack of synchrony between individuals in group B did not permit a simple test of the effect of transferring rams from short days to long days. The individual data in fig. 4.2.7 indicate that in four of the six rams exposed to a prolonged period of short days levels had fallen to long day values by about week 17 on 8L:16D. In the other two rams which still had high levels of plasma β-EP after 22 weeks on 8L:16D, the transfer to long days appeared to induce a rapid decline in β-EP levels.

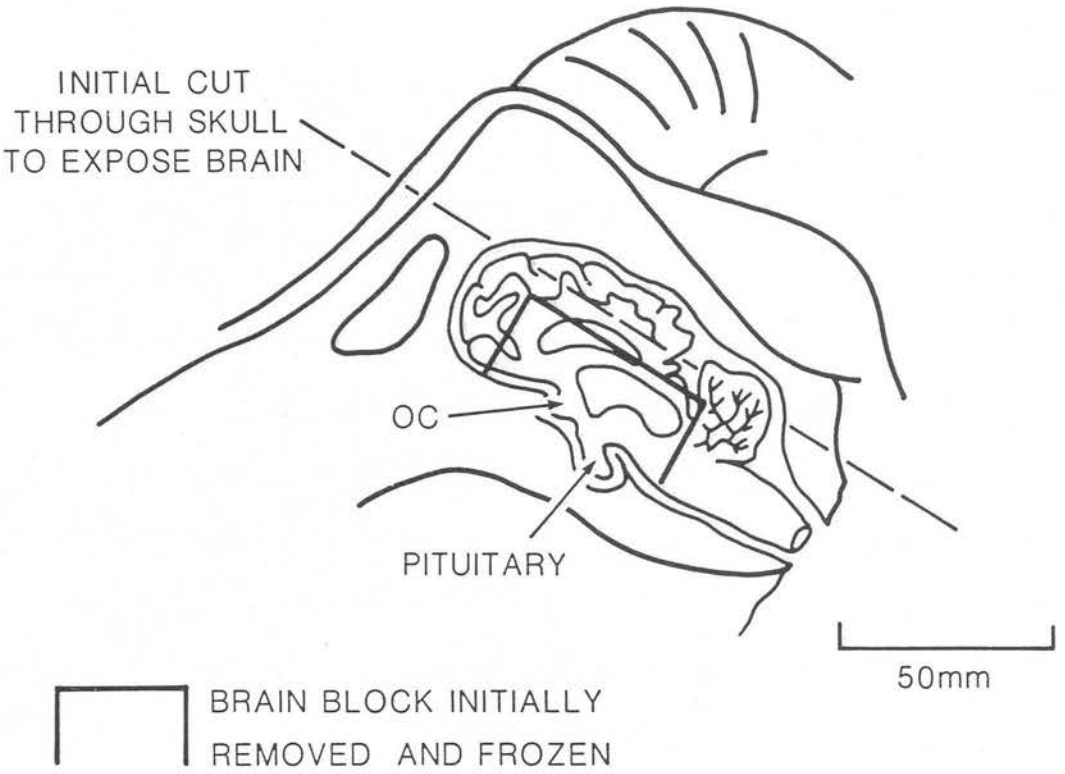

Fig. 4.3.9a

Experiment 10. Sagittal section through skull indicating technique for exposing and removing brain.

4.3 Experiment 10 A study of the distribution of immunoreactive β-endorphin in the hypothalamus of the ram.

4.3.1 Aims

Evidence exists that β-endorphin immunoreactivity in plasma is material released from the pituitary gland rather than from the hypothalamus. β-endorphin is a putative endogenous ligand for mu receptors. The effects of naloxone on LH secretion observed in chapter three indicate an action of naloxone within the hypothalamus. Since naloxone is a high affinity mu receptor antagonist it is possible that the observed responses resulted from blockade of β-endorphinergic inhibition on LHRH release. The aim of this experiment was to investigate whether immunoreactive β-endorphin actually exists within the ram hypothalamus, and to observe whether its distribution resembled that previously observed for LHRH.

4.3.2 Materials and methods

Four intact Herdwick rams and five intact Portland rams were used in this study. The rams had been living outdoors on pasture, and were killed in mid-December by captive bolt gun through the spinal column in the neck. The head was rapidly removed and the brain exposed by a horizontal saw cut through the top of the skull as depicted in figure 4.3.9a. This cut also removed the top 5-10mm of the cerebral cortex. The brain was removed and a crude block of the base of the brain was cut containing the hypothalamus as shown in figure 4.3.9a. The dimensions of the block were approximately 50mm long x 25mm wide x 20mm high. The tissue blocks were rapidly frozen by placing on dry ice in a polystyrene container, and were stored at -40°C until required for sectioning.

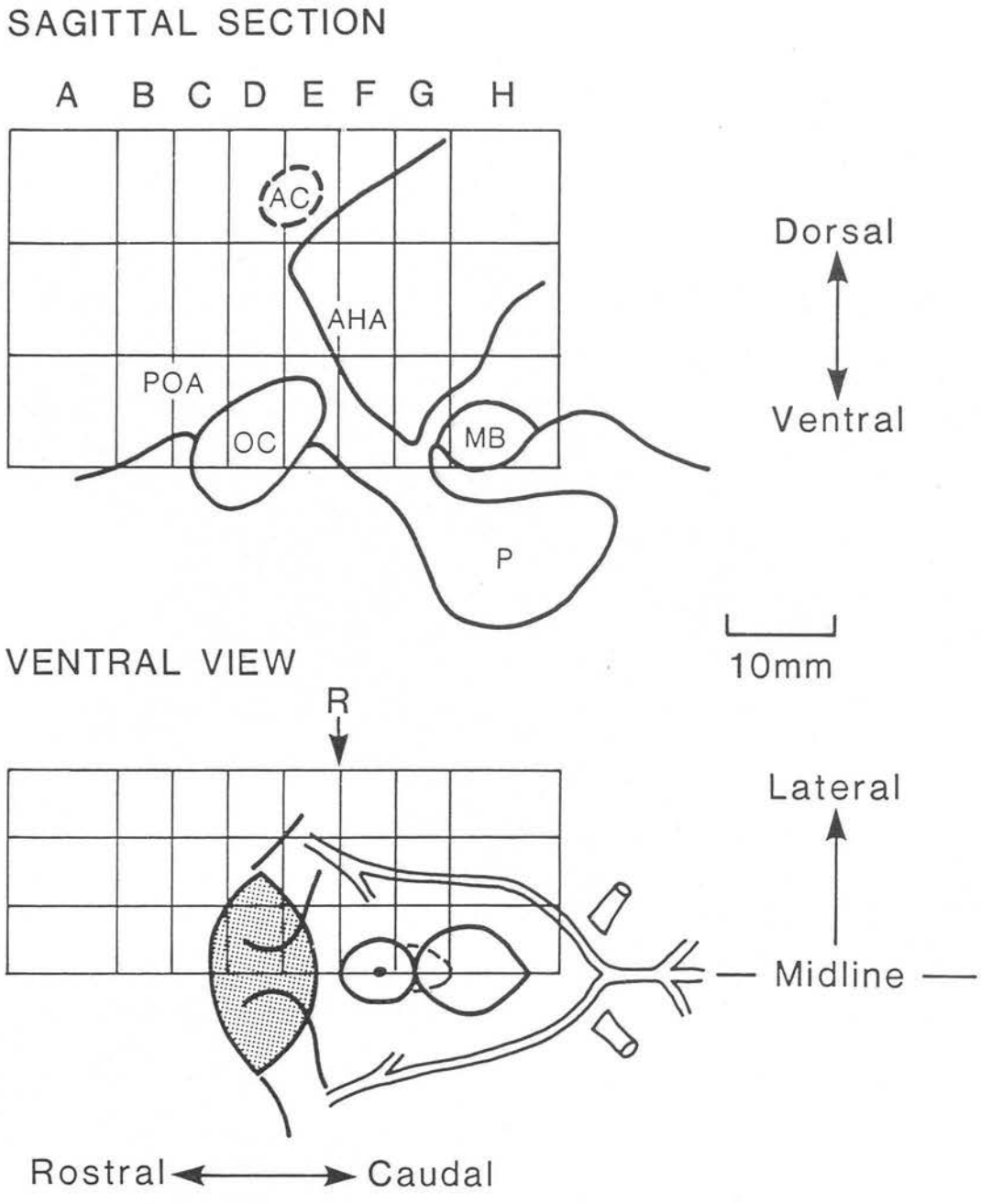

Fig. 4.3.9b

Experiment 10. Sagittal and ventral view of hypothalamic region showing technique for cutting tissue blocks and their location. AC = anterior commissure; AHA = anterior hypothalamic area; MB = mamillary body; OC = optic chiasma; P = pituitary gland; POA = pre-optic area. R = reference point.

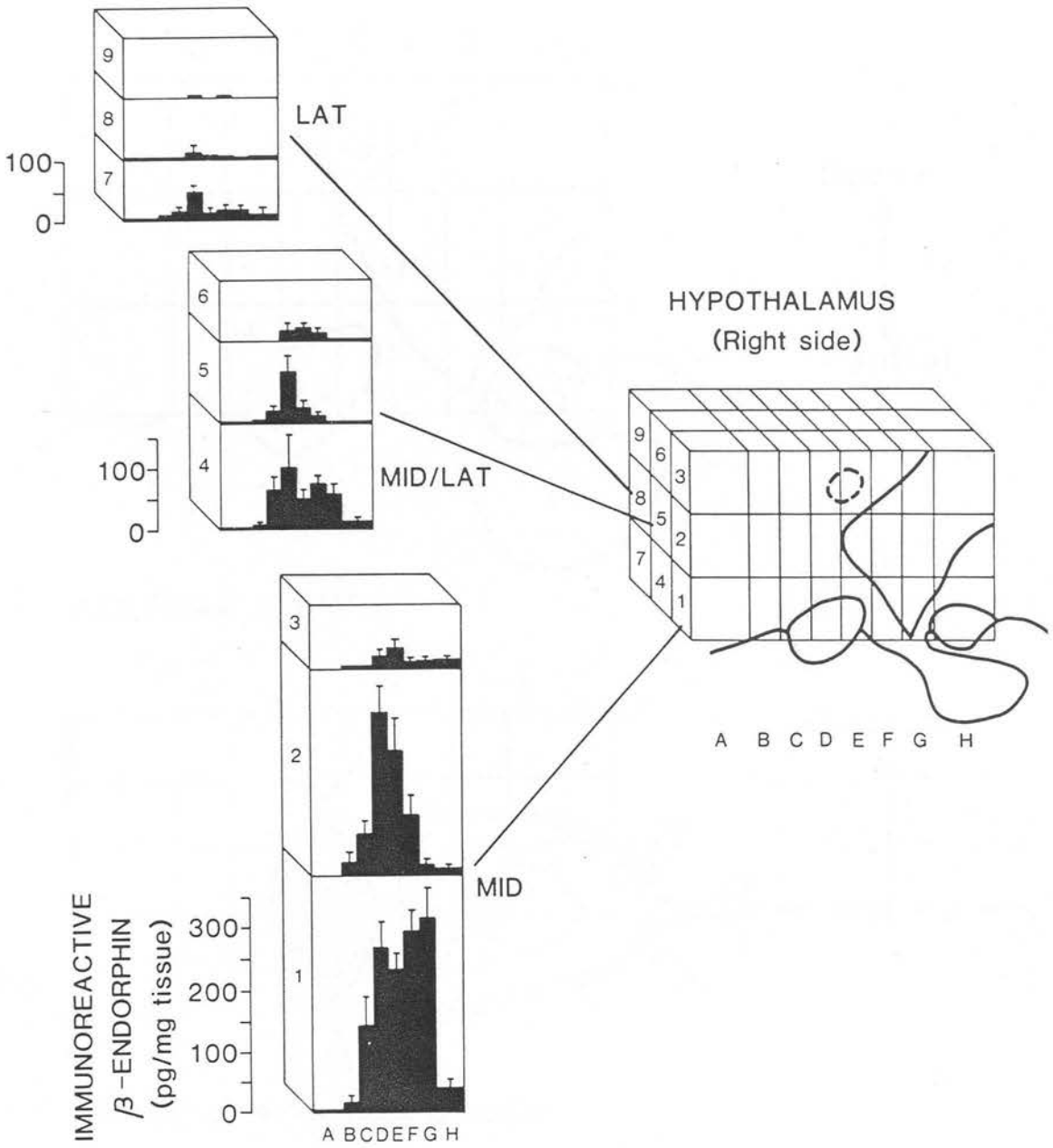

Fig. 4.3.10

Experiment 10. Quantitative distribution of β-EP in hypothalamic tissue blocks from sexually active rams. Each value is the mean ± S.E.M. for 9 rams.

Sectioning was carried out by Dr G.A. Lincoln, based on the techniques of Estes et al. (1977) and Strauss et al. (1979). The tissue block was partially thawed until its brittleness was lost. Using the anterior limit of the pituitary stalk on the ventral surface of the brain as the principal reference point ("R" in figure 4.3.9b) 2.5 mm thick slices of the tissue block were cut by hand along the anterior posterior axis. Slices were cut for 15mm anterior to the reference point, and for 10mm posteriorly. These were split into two down the midline of the brain. One half was kept for later use, the other half was cut into 9 blocks of 3mm lateral width x 5mm height working away from the midline ventral surface of each tissue slice. The location of the 72 tissue blocks generated is indicated in figure 4.3.9b. Tissue blocks were refrozen until required for weighing, extraction and assay as described in chapter 2.1.22

4.3.3 Results

Figure 4.3.10 shows the immunoreative β-endorphin content of each of the 72 blocks expressed in pg per mg of tissue (wet weight). There was a discrete distribution of β-EP, with high concentrations localised in the pre-optic area (blocks C1, C2, D1, D2) and in the median eminence and arcuate nucleas areas (blocks E1, F1, G1). Absolute levels of β-EP were at least 100 fold less than those in pituitary (see experiment 11).

4.4 Experiment 11 Partial characterisation of β-EP in ovine plasma and pituitary extracts.

4.4.1 Aims

Antisera raised against β-endorphin are not highly specific because of the common amino acid sequence with β-lipotropin and various other

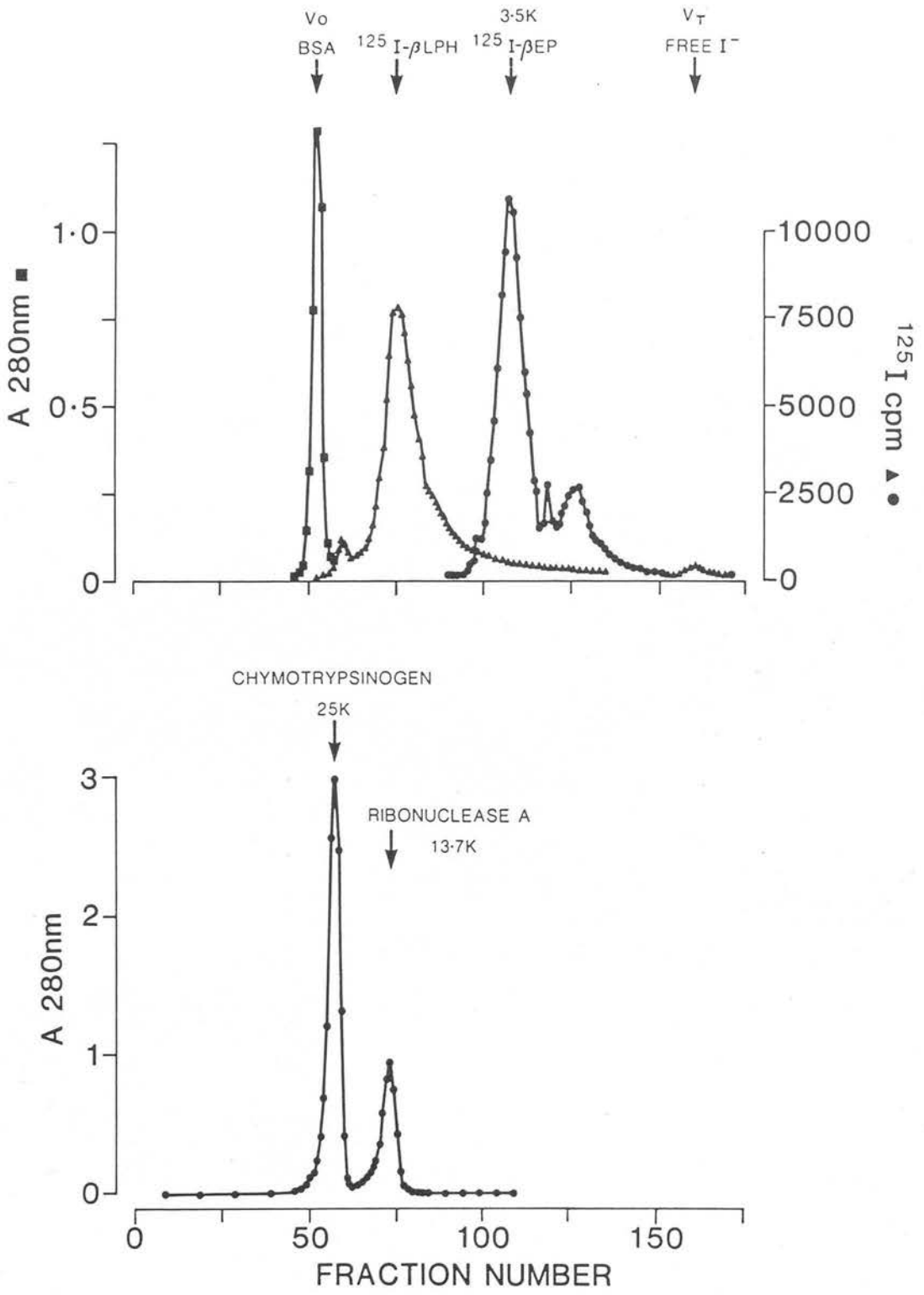

Fig. 4.4.11

Experiment 11. Elution profiles of $\beta_o$endorphin and $\beta_h$lipotropin standards and various calibration markers run on a Sephadex G-50 superfine chromatography column.

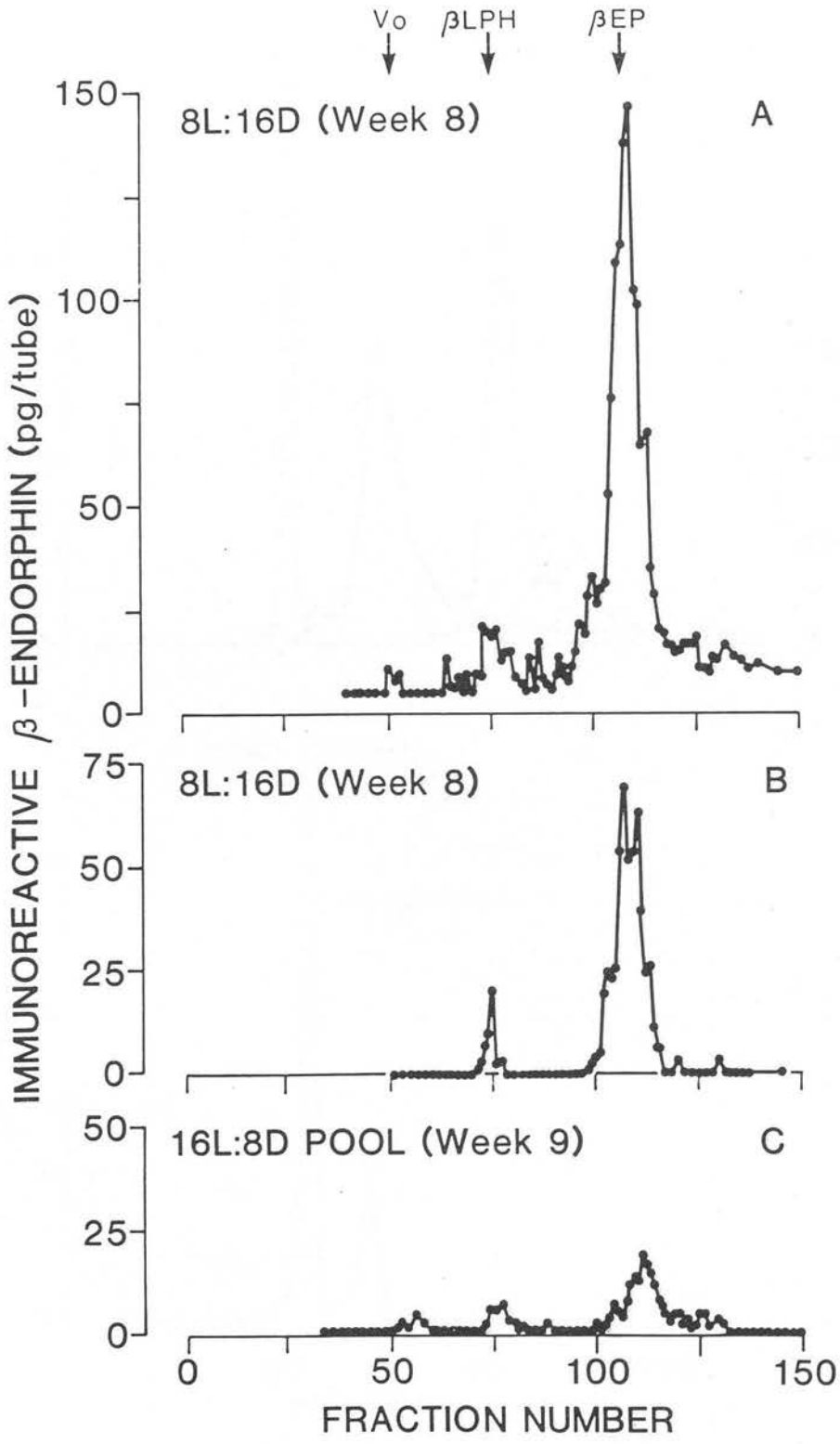

Fig. 4.4.12

Experiment 11. Elution profiles of three separate extracts of fresh plasma run on a Sephadex G-50 superfine chromatography column. Top and middle panels show extracts of plasma taken from rams on a short day photoperiod (8L:16D), and the lower panel shows a pool of extracts of plasma taken from rams on long days (16L:8D).

peptides (see fig. 1.3). The specificities of the two RIA antisera used in the studies described in this chapter are given in table 2.12. The aim of the studies in this section therefore was to partially characterise the immunoreactivity measured by using sephadex gel chromatography to separate cross reacting peptides on the basis of molecular weight.

4.4.2 Materials and methods.

Selected samples collected at the times described in experiments 8 and 9 were VYCOR extracted and chromatographed as described in chapter 2. The plasma samples from experiment 8 were stored for up to one year prior to chromatography whereas those from experiment 9 were extracted immediately (ie. as fresh plasma), and the plasma extracts stored for no more than 14 days before chromatography.

Pituitary glands were collected from the rams killed in experiment 10. These were snap frozen on dry ice and stored at -40°C prior to extraction and chromatography. RIA of column fractions used the 7.9.02 antiserum in all cases.

4.4.3 Results

Figure 4.4.11 shows typical calibration runs using iodinated β-endorphin and β-lipotropin, and using known molecular weight markers. Figures 4.4.12a-c show elution profiles from three separate runs. The vertical axis shows β-EP immunoreactivity in pg/tube. Since the antiserum 7.9.02 has previously been shown to cross-react with β-lipotropin on a 1:1 molar basis with β-EP, the raw measurement of pg/tube also represents the true molar ratio of these two peptides. To correct for real weight of peptide in the plasma the β-lipotropin peak should be multiplied by a factor of approximately 3. Fig 4.4.12a shows

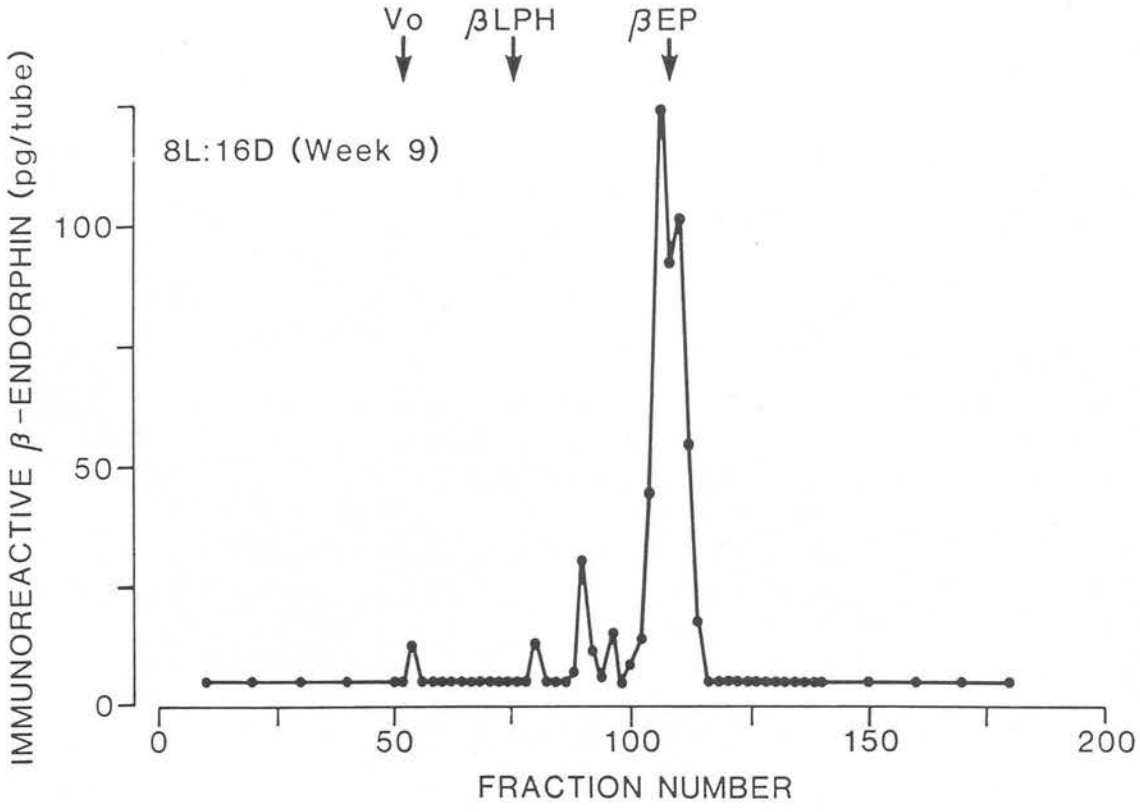

Fig. 4.4.13

Experiment 11. Elution profile of an extract of plasma collected from a ram housed on short days. The plasma was stored at -20°C for nine months prior to extraction and chromatography on a Sephadex G-50 superfine column.

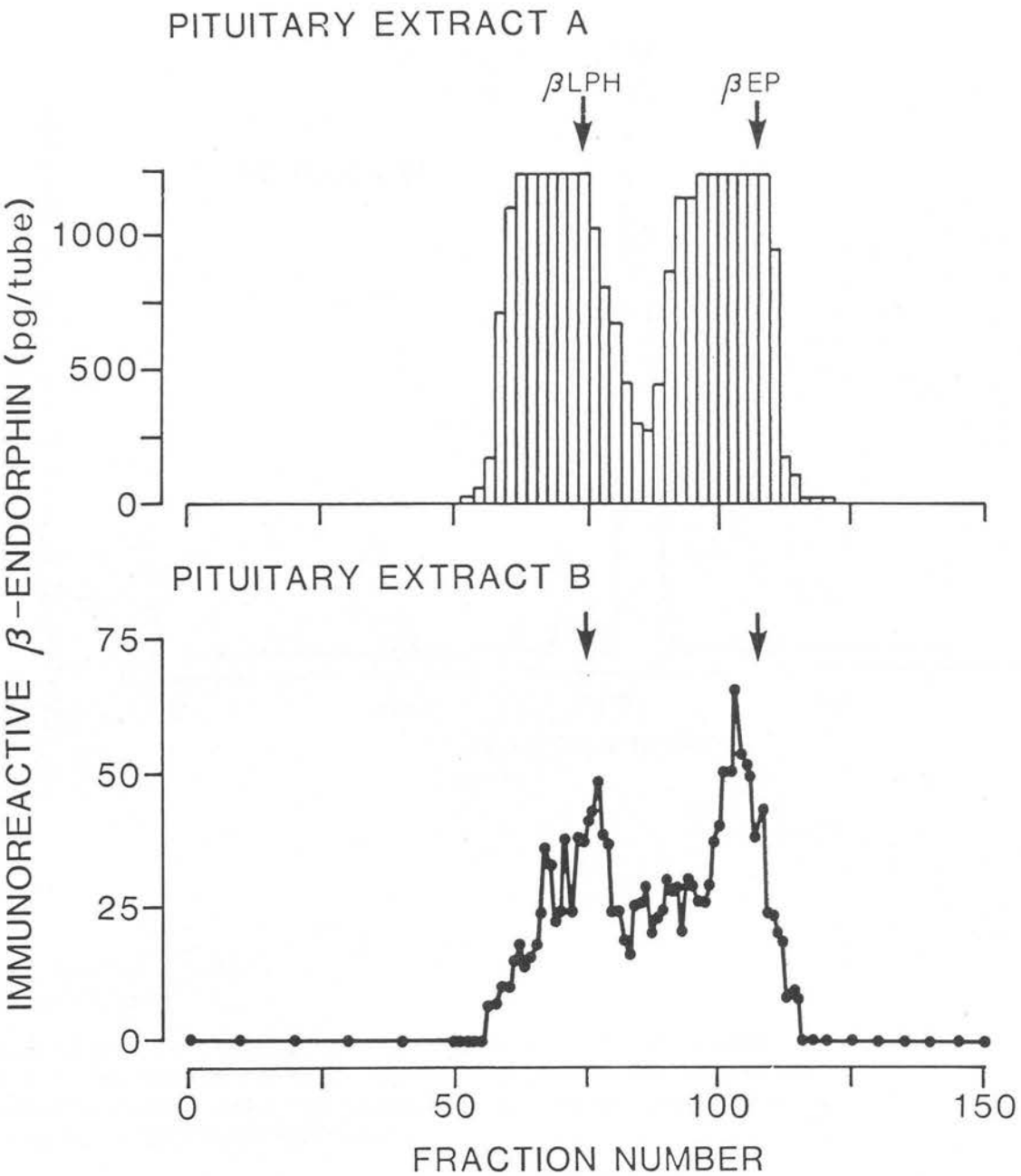

Fig. 4.4.14

Experiment 11. Elution profiles of pituitary extracts from two rams run on a Sephadex G-50 superfine column. The upper panel shows a run using the equivalent of 1/10 of a whole pituitary, and the lower panel shows a run using the equivalent of 1/1000 of a pituitary.

an extract of 25ml plasma extracted fresh from a ram on short days in experiment 9 whose overall level of plasma β-EP was 790pg/ml. The majority of the immunoreactivity in the plasma co-elutes with native β-EP. Fig. 4.4.12b also shows an elution profile of a plasma extract from a ram on short days, again the majority of the immunoreactivity appears to be of the same molecular weight as native β-endorphin, though both animals have a clearly identifiable peak of immunoreactivity eluting in the position of β-lipotropin standard. Fig. 4.4.12c shows a fresh extract of a pool of 60ml plasma from two rams on long days in experiment 9 who had overall β-EP levels of 40-45 pg/ml plasma. The size of the native β-EP peak is much reduced compared to the short day plasmas, however the β-lipotropin peak is still clearly identifiable. Fig. 4.4.13 shows an extract of plasma from a ram on short days in experiment 8 with overall β-EP levels of 650 pg/ml plasma. This plasma was stored for 9 months prior to extraction. The majority of the material co-eluted with native β-EP, however no single peak of immunoreactivity could be seen in the position where β-lipotropin would be expected.

Fig. 4.4.14a-b shows elution profiles of two extracts of whole pituitary glands. In run A (fig. 4.4.14a) the equivalent of 1/10 of the whole pituitary extract was loaded, in run B (fig. 4.4.14b) the equivalent of 1/1000 of a pituitary was loaded. Whole pituitary β-EP content was estimated by multiple serial dilutions, giving a mean value of 39ng/mg tissue. The preliminary data presented in fig. 4.4.14b would suggest that the β-EP present in the pituitary is composed of roughly equal amounts of peptides of the molecular weight of β-lipotropin and those with a molecular weight similar to β-endorphin.

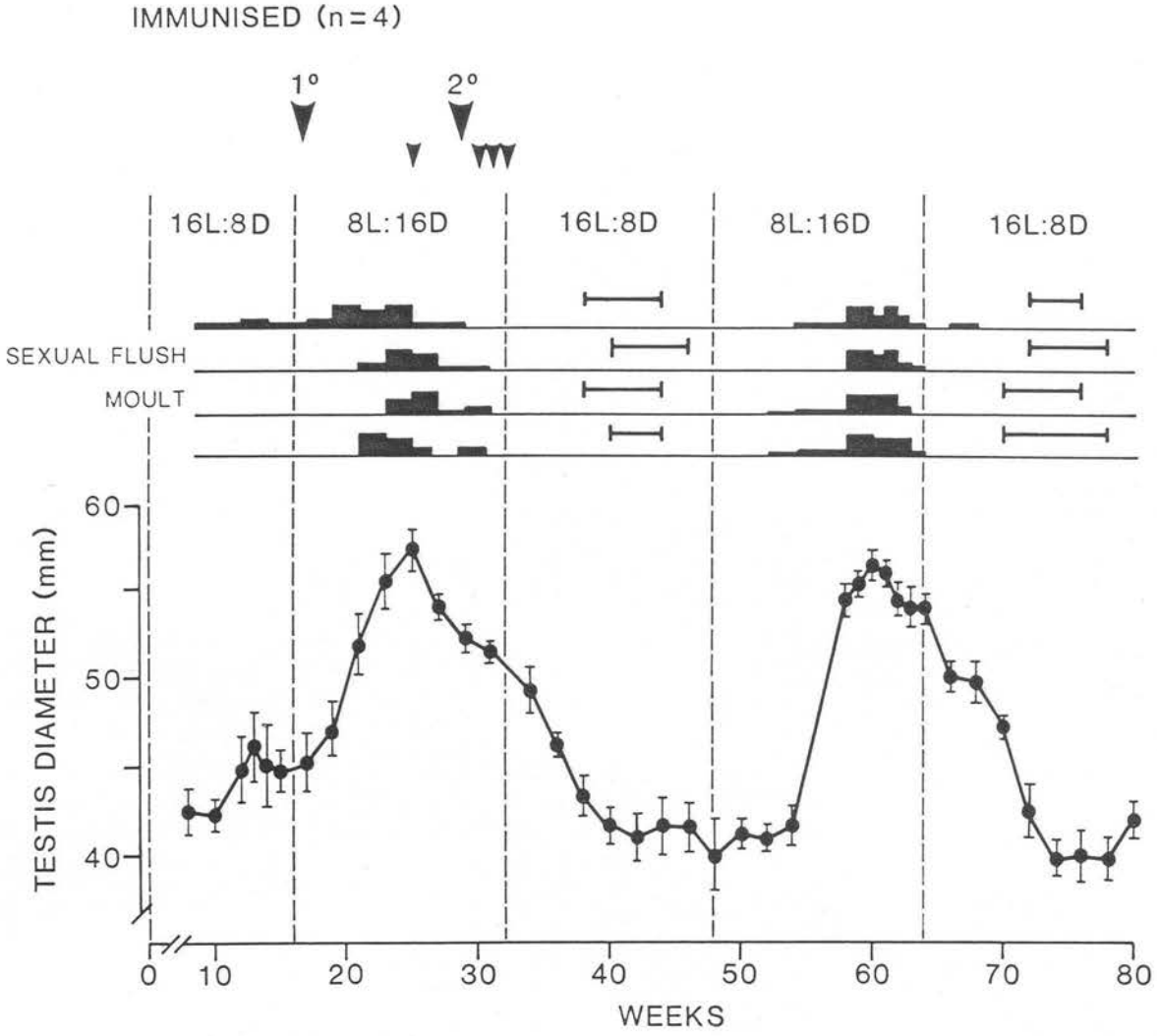

Fig. 4.5.15

Experiment 12. Effect of immunoneutralization of β-endorphin on cycles of testicular diameter, sexual flush, and moulting in four young Soay rams housed indoors under an artificial photoperiod. Values for testis diameter are mean ± S.E.M. ↓ and ↓² respectively indicate the primary and booster immunization. ▼ indicates collection of serum for determination of antibody titres (see table 2.8). ⊢⊣ indicates period of moult, ▬ indicates intensity of sexual flush (arbitrary scale).

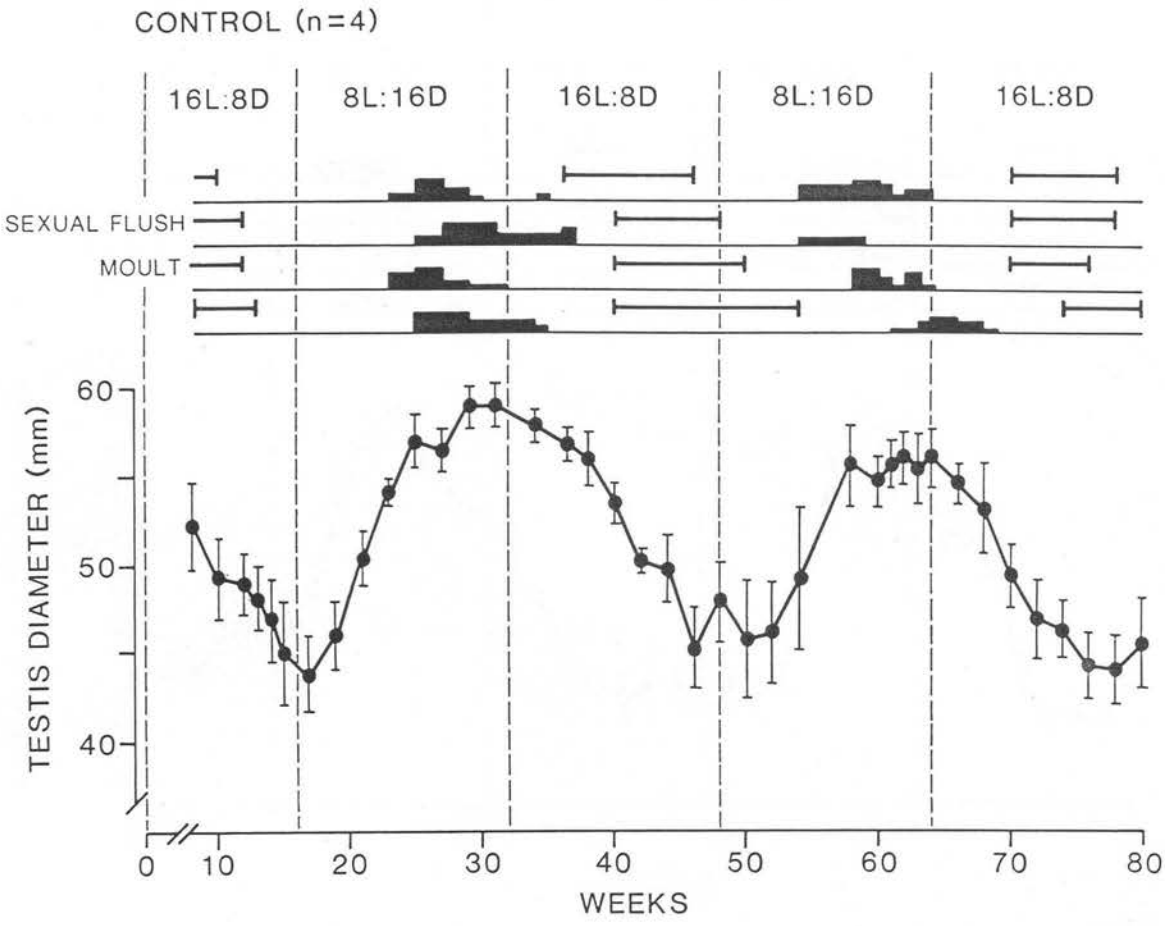

Fig. 4.5.16

Experiment 12. Control cycles of testicular diameter, sexual flush and moulting in four mature Soay rams housed indoors under artificial photoperiod. Values for testis diameter are mean ± S.E.M.

4.5 Experiment 12 Reproductive responses of Soay rams exposed to artificial photoperiods after active immunization against β-endorphin.

4.5.1 Aims

There is a paucity of information on both the relationship, if any, between peripheral EOP levels and the actions of EOP within the brain, and on the physiological functions of EOP in the periphery. The aim of this study was therefore to immunoneutralize circulating β-EP and observe the effects on reproductive and physiological functions.

4.5.2 Materials and methods

This study was carried out in four one year old Soay rams which were housed indoors under artificial photoperiods. Full details of the immunization technique are given in chapter 2.1.8. Observations were made of testis diameter, inguinal skin flush, and moulting during the period of immunization, and similar measurements were made in a group of four mature Soay rams kept in the same room to act as controls.

4.5.3 Results

Fig. 4.5.15 shows the photoperiod treatment, mean testis diameter, and sexual flush and moult in the individual rams. Table 2.8 shows the antibody titres in each ram at the times indicated. Data from the mature control rams are shown in fig.4.5.16. Active immunization against β-endorphin did not appear to affect any of the three parameters studied, in particular the high antibody titres achieved at the end of the first period of short days did not prevent the development of short day refractoriness and the onset of gonadal regression or the continued inhibitory influence of subsequent long day treatment. The reproductive cycle in the immunized rams during the actual period of immunization was clearly very similar

to the subsequent cycle when titres would be expected to have fallen. The differences between immunized and control rams such as the slower rate of testis regression in the controls were likely to have been due to the different ages of the rams in the two groups.

4.6 Discussion

Plasma β-endorphin immunoreactivity

The radioimmunoassay developed for $\beta_o$endorphin compares favourably with the human assays on which the techniques were based, and with other published assays for β-endorphin. Typical assay sensitivity was 5pg/tube. Assuming an extraction efficiency of 60% and a standard extraction volume of 3ml plasma the limit of detection in the current assay system is under 10pg/ml plasma. In the original human assay the limit of detection for a 3ml sample was 15pg/ml (Jeffcoate et al., 1978a). Other published assay sensitivities are 50pg/tube (Guillemin et al., 1977a), 25pg/tube (Wardlaw and Frantz, 1979), 20pg/tube (Höllt and Bergmann, 1982) and 10pg/tube (Ghazarossian et al., 1980). The levels of immunoreative β-EP under long days (typically 20 - 100pg/ml) are comparable to those observed in normal human subjects (Wardlaw and Frantz, 1979; Ghazarossian et al., 1980; Smith et al., 1981; Panerai et al., 1982), and those under short days (250pg/ml) are comparable to those in normal rat plasma (Höllt et al., 1978; Petraglia et al., 1982).

The specificities of the two antisera used are also comparable to polyclonal antisera previously described in that they show significant cross reactivity with the β-lipotropin precursor. The antiserum B4.2 raised against porcine β-endorphin would appear to specifically recognise the c-terminal sequence (residues 27 - 31) since there is a poor and non-parallel cross reactivity with human β-EP and β-LPH

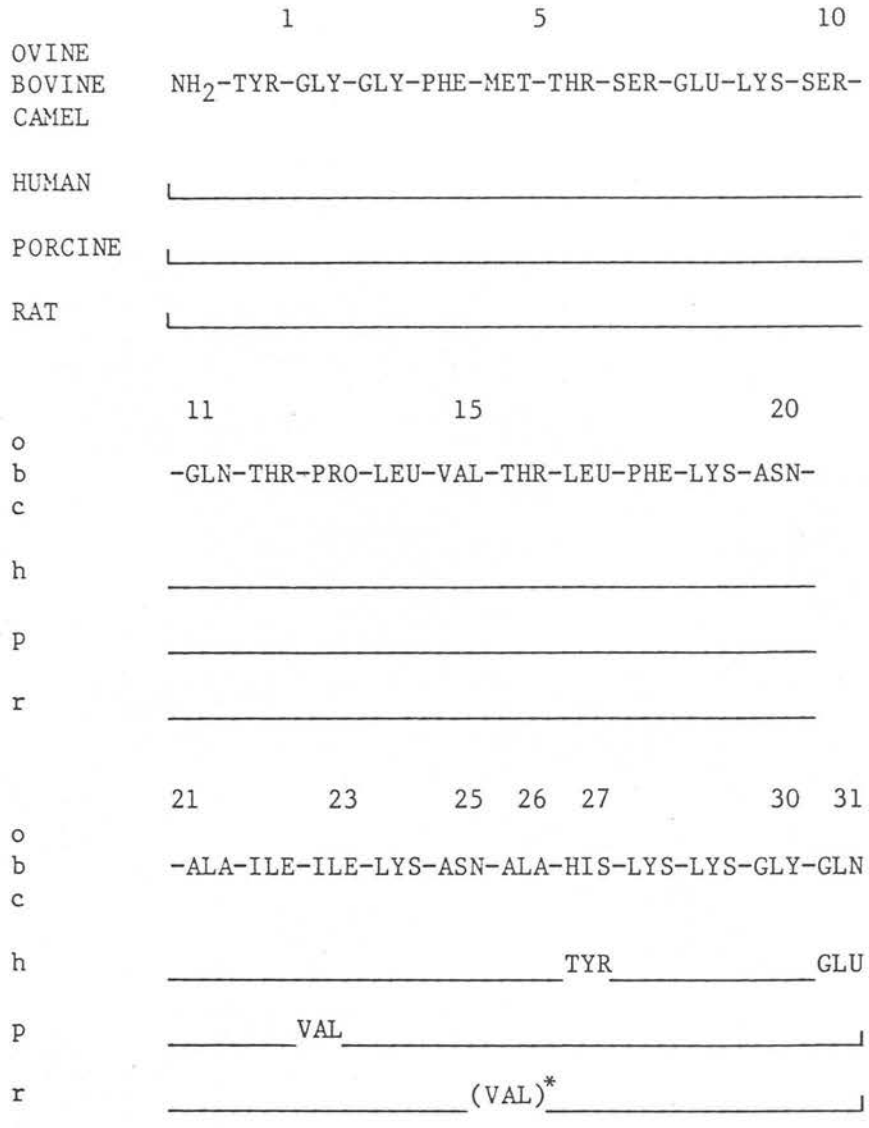

Figure 4.6.17

Amino acid sequence of B-endorphin$_{1-31}$(= B-lipotropin$_{61-91}$) from different species. Based on data from Li, (1981); Rubinstein et al. (1977); Seidah et al. (1977).
* Based on cDNA sequencing data (Drouin and Goodman, 1980).

which differ in this region (see Fig. 4.6.17). This non-parallelism of human peptides in the B4.2 assay makes it difficult to quantitatively assess cross reactivity with acetyl and non-acetyl β-EP_{1-27} which contain the human sequences (see table 2.12). This antiserum showed negligible cross-reactivity with N-terminal sequences even though these are identical in ovine, porcine and human β-EP. Certain studies have previously shown non-parallelism between β-EP from different species (Wardlaw and Frantz, 1979; Wilkes et al., 1980b).

After the completion of experiment 8, all studies used the 7.9.02 antiserum. The initial reason for change was the very limited supply of antiserum B4.2, however the 7.9.02 homologous RIA had several advantages, for example the working range of the standard curve and the sensitivity were greater, and a more valid estimate of specificity could be made since human peptides showed parallel inhibition of ^{125}I-$\beta_o EP$ tracer binding to antiserum 7.9.02 with the ovine standards. The antiserum 7.9.02 also appears to recognise the C-terminal region, though does not discriminate the glutamine/glutamic acid substitution in position 31. The C^1-fragment (β-EP_{1-27}) cross reacts significantly, and acetylation at the N-terminal only marginally reduces crossreactivity. None of the N-terminal peptides cross react significantly. The apparent crossreactivity of dynorphin is very likely to be artifactual, resulting from contamination of dynorphin with a small amount of β-EP standard, since dynorphin only shares a common peptide sequence with Leu-enkephalin and the four N-terminal residues of β-EP_{1-31}. Met-enkephalin showed no detectable cross reactivity with 7.9.02 even in a 10^6 fold excess.

The seasonal variation in plasma β-endorphin immunoreactivity observed in experiment 9 is one of the few demonstrations that peripheral β-EP levels change under physiological circumstances.

Several studies have concentrated on altered plasma β-EP levels in pathological states and pharmacological manipulations of the adrenal axis because β-EP is derived from a common precursor with ACTH and possibly secreted concomitantly with ACTH from anterior pituitary corticotrophs (see chapter 1.2.8). Two studies have demonstrated increased β-EP levels during labour in women (Fletcher et al., 1980; Goland et al., 1981), and increased plasma β-EP levels are associated with many stressful and painful procedures (Holaday and Loh, 1981). One study of plasma β-EP in normal women failed to demonstrate convincing physiological changes during the menstrual cycle, though the authors claimed that a preovulatory peak occurred two days prior to the LH surge (Vrbicky et al., 1982). The authors do not report the actual levels of plasma β-EP or the magnitude of the supposed pre-ovulatory peak. The current study, however, has clearly shown that plasma β-EP levels change by 5 - 20 fold during photoperiodically driven reproductive and metabolic cycles in Soay rams. The physiological significance of this will be considered later.

Sephadex chromatography indicates that the majority of the immunoreactivity in plasma is β-endorphin, and the seasonal change in total immunoreactivity is mainly due to a change in β-endorphin; β-lipotropin levels change at most by only a 2-fold rise. It is interesting to note that an ovine β-lipotropin radioimmunoasay was developed several years before the discovery of β-endorphin and the enkephalins (Desranleau et al., 1972), however studies on plasma β-lipotropin were not pursued, perhaps because levels were very low.

It is suprising that no evidence of circadian rhythmicity was found. In human subjects circadian rhythmicity has been observed in plasma β-lipotropin levels (Jeffcoate et al., 1978b; Mullen et al.,

1979) and in total plasma β-EP immunoreactivity (Dent et al., 1981; Lim and Funder, 1983). Levels peak in the late night/early morning, and show a good correlation with adrenal axis function as indicated by plasma ACTH levels (Mullen et al., 1979) or plasma cortisol levels (Dent et al., 1981). However cortisol rhythms in ruminants are not as clearly defined as those in humans (Wagner and Oxenreider, 1972; McNatty et al., 1972; Lincoln et al., 1982), thus if plasma β-EP levels are associated with adrenal function circadian rhythmicity might not be evident. The hourly sampling regime may not be adequate to define subtle circadian fluctuations. Fulkerson and Tang (1979) sampled three merino ewes at 10 minute intervals for 24 hours and demonstrated that cortisol was secreted in short episodes, thus ultradian rhythmicity may mask underlying circadian rhythmicity. In the present study , β-EP levels were extremely constant within individuals, and it seems quite possible that β-EP secretion in sheep is not associated directly with adrenal function. The current sephadex chromatography studies indicate that the proportion of β-endorphin to β-lipotropin is much higher in plasma than in whole pituitary extracts. These pituitary extracts probably represent mainly the anterior lobe ratio, however sheep also have a small neurointermediate lobe. Several lines of evidence indicate that differential processing of pro-opiomelanocortin occurs in the two lobes (see chapter 1.2.8). In the neurointermediate lobe β-EP is produced with β-MSH and very little β-lipotropin is formed, whereas higher levels of β-lipotropin are found in the anterior lobe where β-EP is produced with ACTH. The ratio of β-endorphin to β-lipotropin in ovine plasma might therefore indicate that a substantial proportion of β-EP is derived from the neurointermediate lobe. Some direct evidence for this hypothesis exists (Clarke et al., 1983, 1984).

Hypothalamus-pituitary disconnection in the ewe results in a hypertrophied neurointermediate lobe and a significant increase in plasma β-endorphin levels. This increase can be blocked by a dopamine agonist without altering ACTH levels, thus supporting earlier findings that neurointermediate lobe secretion was controlled by dopaminergic inhibition whereas anterior lobe release of pro-opiomelanocortin products is under neurohumoral control (see chapter 1.28; Vermes et al., 1980; Sharp et al., 1982).

The very high levels of immunoreactive β-EP observed in the sheep pituitary in the current study and in the rat and human pituitary (Zakarian and Smyth, 1979; Wilkes et al., 1980a; Höllt and Bergmann, 1982) support the conclusion that peripheral β-EP levels are the result of pituitary secretion, however, the possiblity exists that the observed seasonal changes in plasma β-EP may represent extra-pituitary secretion. The adrenal gland is a major site of met-enkephalin secretion (Clement-Jones et al., 1980) but no evidence suggests that β-endorphin is secreted from this gland. Since the seasonal pattern of β-EP correlated with the pattern of testicular activity it might be suggested that the testis secretes β-endorphin. Authentic β-endorphin has been detected in semen and whole testis extracts from humans and rats, but the low levels found indicate a paracrine rather than endocrine function (Sharp and Pekary, 1981; Margioris et al., 1983). Measurements of immunoreactive β-endorphin in rat testis interstitial fluid indicated levels that were 50 - 100% higher than levels in corresponding rat plasma (Ebling and Sharpe R.M., unpublished results), but this difference would not seem sufficient to indicate secretion of β-endorphin. One study in rats suggests that castration does not alter plasma β-EP levels in the short term though a 50% reduction was observed 5 weeks post operatively, (Petraglia et al., 1982). These

observations indicate that plasma β-EP is not of gonadal origin, but the authors suggest that gonadal steroids might modulate release of β-EP since testosterone replacement therapy in castrate rats partially restored β-EP levels in plasma and anterior pituitary. There are conflicting reports, however, on the interaction of gonadal steroids and β-endorphin secretion. Lim and Funder (1984) observed that ovariectomy actually increased plasma β-EP in rats, and that oestrogen therapy reversed this increase in a dose dependent fashion. In no studies have the effects of gonadal steroids been as marked as the effects of adrenal steroid manipulations (see chapter 1.2.8).

The seasonal pattern of plasma β-EP in rams suggests that β-endorphin may have a endocrine function. It is interesting to speculate what this physiological role might be. The data in chapter 3 indicate that EOP in the hypothalamus inhibit LHRH release, so the question arises as to whether peripheral EOP might have a central action. The low permeability of β-endorphin across the blood-brain barrier would tend to exclude this hypothesis, though the possibility remains of a neuromodulatory function within the median eminence. As previously discussed in chapter 3.8, parenteral administration of β-endorphin can alter neuroendocrine function, however the plasma levels produced by such treatments are very considerably higher than normal physiological levels (Catlin et al., 1981; Reid et al., 1981). In experiment 11 no obvious effects on the reproductive axis of rams were observed following immunoneutralization of good antibody titres. Previous studies using passive immunization of β-endorphin in the circulation of rats also failed to show any neuroendocrine effects (Tannebaum et al., 1979). In a passive immunization study where the anti-β-endorphin serum was injected directly into the arcuate nucleus

in the brain a significant increase in LH secretion occured (Schulz et al., 1981). These observations do not support the hypothesis that peripheral β-EP has central inhibitory effects on LH secretion.

It has been observed in humans that naloxone increases pituitary secretion of pro-opiomelanocortin derived peptides such as ACTH and β-lipotropin and that endogenous and exogenous opiates can suppress plasma ACTH and cortisol (Stubbs et al., 1978; Ropert et al., 1981; Gaillard et al., 1981; Grossman and Besser, 1982). It is possible that β-endorphin may act as a short-loop feedback mechanism in the adrenal axis, perhaps by an effect on corticotrophin releasing factor secretion in the median eminence. A second study by Grossman et al. (1982b) indicated that ACTH responses to naloxone were similar at all stages of the diurnal rhythm in adrenal function. The authors therefore concluded that although EOP mechanisms tonically inhibit ACTH release they are not causal in generating the circadian rhythm, thus it might seem unlikely that they induce seasonal cycles in adrenal activity. Seasonal changes in adrenal function in sheep are poorly understood. Several studies have failed to demonstrate seasonal changes in plasma cortisol levels or any effects of photoperiodic manipulations such as pinealectomy on cortisol secretion (Barrell and Lapwood, 1978; Kennaway et al., 1981; Lincoln et al., 1982). However, a recent study demonstrated a seasonal rhythm in cortisol secretion in White-tailed deer stags with peak levels in the autumn (Bubenik and Leatherland, 1984). The authors speculate that this increase in adrenal activity may pre-adapt the stags to the metabolic stresses of the rut. Since this would correlate with the rise in plasma β-EP currently observed in the Soay ram when transferred to short days it would be premature to rule out a role for plasma β-EP in adrenal function.

Rhythms in nutritional status are very pronounced in semi-domesticated sheep breeds and deer. Body weight increases during the spring and summer as fat reserves are laid down, and the autumn rut is associated with a marked loss in appetite and utilisation of stored fat (Kay, 1979). These changes are photoperiodically controlled rather than simply linked to food quality in the environment (Brown et al., 1978; Argo and Kay, 1984; see also chapter 5). The correlation between elevated peripheral levels of β-endorphin and increase in metabolism of stored fat reserves supports the hypothesis of Margules (1979) that β-endorphin is an important lipolytic hormone. It is interesting to note that Li (1964) proposed the name "lipotropin" because of the lipolytic activity of this peptide in bioassay systems in vitro. Several other anterior pituitary hormones also show lipolytic activity in this bioassay, thus it seemed likely that stimulation of lipolysis was not a specific function. Interest in lipolytic functions has re-emerged with the discovery that the lipolytic peptides were derived from a common precursor. Using a rabbit adipocyte in vitro bioassay four sequences in the pro-opiomelanocortin molecule have been identified as possessing lipolytic activity: $ACTH_{1-10}$, β-MSH, the C-terminal region of β-endorphin, and a N-terminal region of pro-opiomelanocortin (Richter and Schwandt, 1983). β-endorphin has considerably greater lipolytic activity than other endogenous opioid peptides (Jean-Baptiste and Rizack, 1980). These studies have demonstrated that lipolytic actions are mediated via cyclic-AMP mechanisms, but there is some doubt as to whether opiate receptors are actually involved since naloxone does not seem to block endorphin-stimulated fatty acid release in vitro (Schwandt et al., 1979; Jean-Baptiste and Rizack, 1980).

A recent *in vivo* study in rabbits has demonstrated that β-endorphin increases plasma levels of free fatty acids and glycerol, and this can be blocked by naloxone (Richter *et al.*, 1983). Several other observations support a link between peripheral β-EP levels and adipose tissue metabolism, for example elevated levels are found in genetically obese mice and rats (Margules *et al.*, 1978) and in obese women (Givens *et al.*, 1980), and morphine can induce hyperglycaemia in rats (Feldberg and Shaligram, 1972). No studies have been conducted on β-endorphin and lipolysis in sheep, so the question remains open. It is also possible that β-endorphin may have direct effects on pancreatic insulin release. Immunoreactive β-endorphin has been identified in the pancreas (Bruni *et al.*, 1979), and *in vitro* studies using a perfused dog pancreas indicate that β-endorphin and morphine increase insulin and glucagon secretion (Ipp *et al.*, 1978). Complimentary studies by Reid and Yen (1981) demonstrated that β-endorphin given to healthy adult humans elevated plasma glucagon and insulin and hence glucose levels.

The high peripheral concentrations of β-EP observed under short days also correlated with hyperaemia of the inguinal area - the "sexual flush". It has been suggested that EOP are potent vasodilators in peripheral areas. The mechanism by which the sexual flush is produced is not fully understood. It is presumably an autonomic response,but also requires a permissive testosterone environment (Lincoln, 1984). It is possible that plasma β-EP also participates in this response. A study by Lightman *et al.* (1981b) suggested that naloxone could reduce climacteric flushing in post-menopausal women, however a recent study by DeFazio *et al.* (1984) failed to confirm this finding. These studies are difficult to interpret since a possible peripheral effect of naloxone might be confused with a central effect on LH release.

Increased LH secretion is itself associated with an increase in menopausal flushes (Casper et al., 1979). Administration of met-enkephalin analogue to normal human subjects can induce facial flushing (Stubbs et al., 1978), and the effects of opiates on thermoregulation appear to be similar to the physiological changes which accompany hot flushes (Molnar, 1975; Meldrum et al., 1979), thus the role of plasma β-EP in the sexual flush in rams is worth further investigation.

Hypothalamic β-endorphin immunoreactivity

The studies described in chapter 3 indicate that EOP exert a tonic inhibitory influence on LHRH secretion from the hypothalamus. Naloxone is a high affinity mu receptor antagonist, but at sufficient doses also appears to be able to effectively antagonize other receptor subtypes (Pfeiffer and Herz, 1984), thus the techniques used in chapter three do not directly indicate which EOP are actually involved. The aim of experiment 11 was to adapt the RIA techniques previously developed for measurement of β-EP in plasma so that studies could be pursued on the distribution of β-endorphin/β-lipotropin within the hypothalamus. The extraction technique for hypothalamic tissue is based on a combination of published procedures. The assay is a semi-direct method, but the high concentrations of β-EP present allows most of the non-specific assay interference to be diluted out. Whereas β-endorphin-like immunoreactivty is stable in plasma, it is relativly unstable in brain homogenates, so the boiling step is important. Even after boiling some proteolytic activity persisted, so the technique principally allows comparisons of β-EP from different brain areas assuming that proteolytic activity does not vary significantly.

The results indicate that high concentrations of β-EP occur in

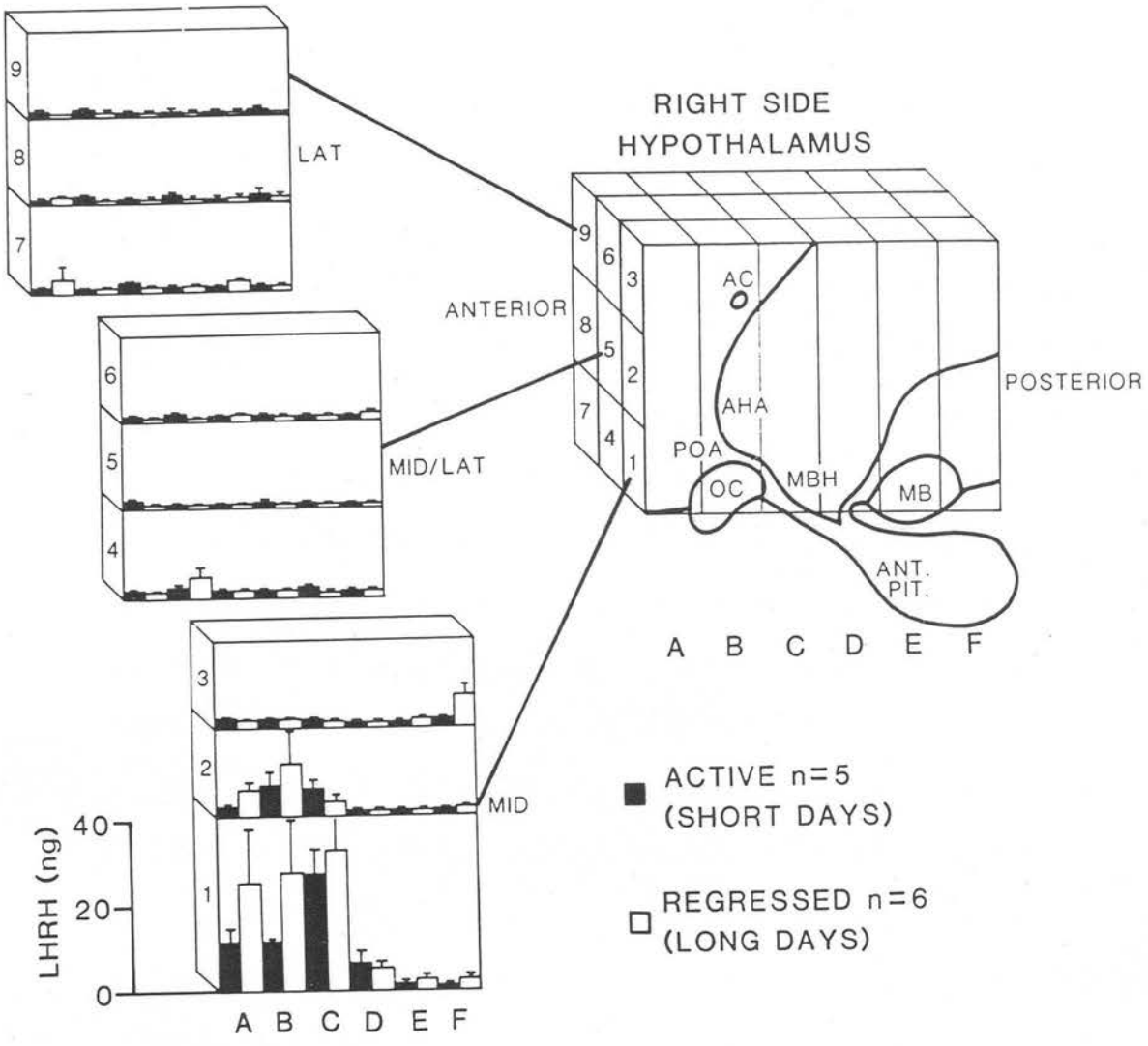

Fig. 4.6.18

Quantitative distribution of LHRH in hypothalamic tissue blocks from sexually active and inactive rams. Data from Lincoln et al. (1985).

AC - anterior commissure; AHA - anterior hypothalamic area; ANT.PIT. - anterior pituitary gland; MB - mamillary bodies; MBH - medio-basal hypothalamus; OC - optic chiasma; POA - pre-optic area.

both the median eminence/arcuate nucleas area and the pre-optic area. This distribution is similar to that observed by G.A. Lincoln and H.M. Fraser (unpublished results) for LHRH. Their study used the same sectioning technique, and the results are shown in fig. 4.6.18. The tissue block technique does not provide such an accurate localisation of peptides relative to anatomical nuclei as the punch methods developed in rat brain, however it has a number of advantages. Firstly, in sheep the stereotaxic location of specific nuclei can vary by several millimetres (McKenzie and Smith, 1973), and secondly immunohistochemical analysis of LHRH distribution demonstrate that distribution of the cell bodies is not restricted to specific hypothalamic nuclei and tracts. The correlation between LHRH and β-EP distribution provides indirect evidence that endorphinergic mechanisms may affect LHRH secretion. Support is equally given for a direct neuromodulatory role on LHRH release from axon terminals in the median eminence and for neuromodulatory roles on neural imputs to LHRH cell bodies at a higher level. The significance of β-EP associated with LHRH in the pre-optic area is not clear. Several studies in the ewe suggest that lesions in the anterior hypothalamus prevent the oestrogen induced LH surge, but do not affect basal LH secretion (Jackson et al., 1978; Radford, 1979; Przekop and Domanski, 1980). These observations suggest that preoptic area neural inputs are not required to maintain tonic pulsatile LH secretion in the ewe. Comparable studies on anterior hypothalamic les ions have not been carried out in rams, thus it is open to speculation that preoptic area inputs might be involved in seasonal changes in LH pulse frequency and that the high levels of β-EP reflect modulatory actions of EOP on seasonal LHRH synthesis and release in this brain region.

The levels of immunoreactivity measured in the current study are comparable to those previously reported in rat, porcine, human and primate hypothalami. The antiserum used in the current studies cross-reacts to a relativly high degree with non-active acetylated forms of β-endorphin, so the actual physiological significance of hypothalamic β-endorphin-like immunoreactivity cannot be directly gauged. However recent studies in both the rat and the rhesus monkey indicate that $\beta\text{-EP}_{1-31}$ is the predominant form in the hypothalamus (Zakarian and Smyth, 1982; Akil et al., 1983). This is in contrast to other brain areas such as the hippocampus and brain stem where N-acetyl-$\beta\text{-EP}_{1-27}$ and N-acetyl-$\beta\text{-EP}_{1-26}$ are predominant; forms which have little opioid activity. Further studies are necessary to demonstrate whether a similar distribution of active and inactive forms occurs in sheep, however if it could be demonstrated that the B-endorphin immunoreactivity observed in the current study is biologically active and varies in relation to season this would provide good supporting evidence that β-endorphinergic mechanisms inhibit LHRH in the hypothalamus.

4.7 Summary

1) Immunoreactive β-endorphin (β-EP) is readily detectable in ovine plasma. There is a pronounced seasonal variation; under short day photoperiods levels rise by approximately 10-fold.

2) No circadian rhymicity in plasma β-EP is detectable, either under long days when mean levels are low (50pg/ml) or under short days when mean levels are high (250pg/ml).

3) The predominant constituent of plasma β-EP immunoreactivity is either native β-endorphin or forms of a very similar molecular size. Seasonal changes in circulating β-endorphin levels appear to be much greater than changes in β-lipotropin levels.

4) Substantial amounts of immunoreactive β-endorphin are found in the ovine hypothalamus, and are associated with both the median eminence/arcuate nucleus area and the preoptic area. This distribution is similar to that previously observed for LHRH, and supports the hypothesis that β-endorphin may be involved in the physiological mechanisms governing hypothalamic LHRH release.

Chapter 5

Generation of melatonin rhythms and their role in relaying effects of photoperiod on reproduction and seasonal physiological cycles.

Generation of melatonin rhythms and their role in relaying effects of photoperiod on reproduction and seasonal physiological cycles.

5.1 Aims.

The first aim of these experiments was to study plasma melatonin rhythms in rams following exposure to prolonged periods of constant light and dark and to relate these to the reproductive and somatic responses of the rams in an attempt to clarify the role of melatonin rhythms in photostimulation and photorefractoriness. Previous studies have indicated that over short periods melatonin rhythms in sheep will free run under constant dark (Rollag and Niswender, 1976) and constant light (Almeida and Lincoln, 1984b), however these studies were not of sufficient duration to determine the effects of such treatments on the reproductive axis. The second aim was to use the sheep maintained under prolonged constant conditions to investigate the role of light/dark cycles in the generation of melatonin rhythms. As reviewed in chapter 1, it has been suggested that light has a dual effect on pineal melatonin rhythms: a direct suppressive effect, and an indirect entrainment effect. The current studies were designed to further investigate this hypothesis by testing the effects of one hour light or dark pulses on plasma melatonin patterns in sheep previously maintained on constant dark or constant light.

5.2 Materials and methods.

The design of the experiment is summarised in figure 5.1. Sixteen intact Soay rams of 16 months age at the start of the experiment were studied. Three months prior to the start of the experiment the rams were moved into indoor lightproof housing and kept on a controlled photoperiod of 16L:8D, thus allowing the rams to acclimatise to their

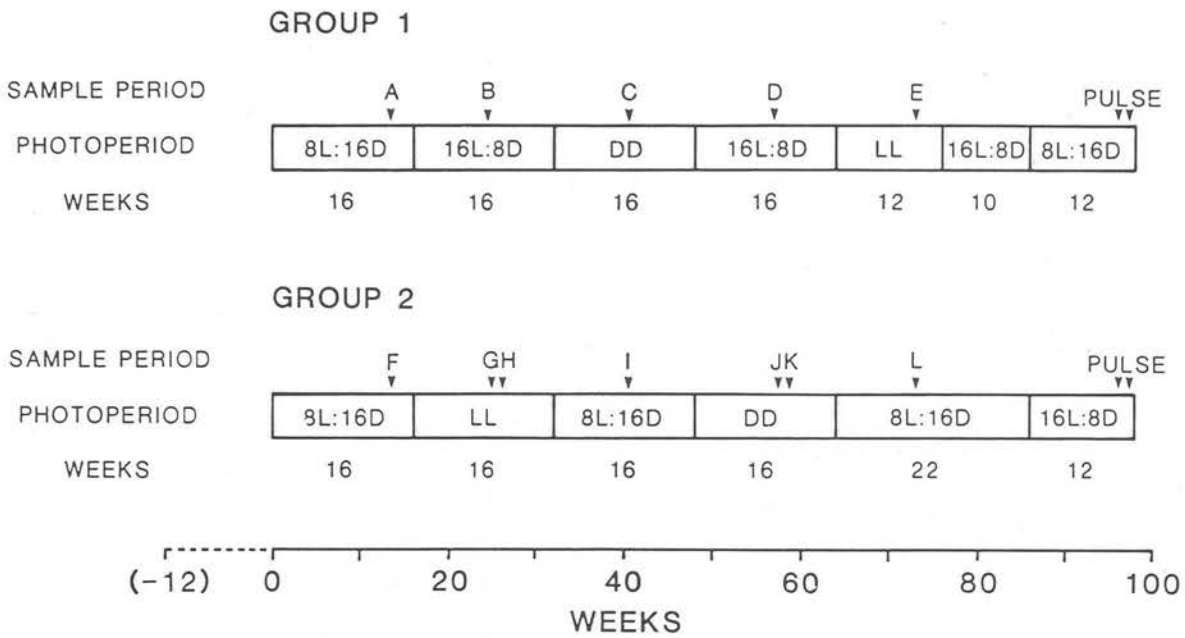

Fig 5.1

Experimental design for investigating the effects of constant illumination (LL) and constant darkness [=dim red light] (DD) on reproductive and somatic cycles and plasma melatonin rhythms. The letters A to L indicate occasions when hourly blood samples were collected for 50 hours for measurement of plasma melatonin. Weekly blood samples were also collected for measurement of plasma FSH and prolactin levels, and weekly or biweekly measurements of testis diameter, sexual flush, moult stage and food intake were made. PULSE indicates the period when experiment 5.3.4 was carried out. In the "PULSE" experiment melatonin rhythms were studied in rams transferred from the photoperiod indicated to a skeleton photoperiod consisting either of a single one hour light period per 24 hours darkness or of two x one hour light periods per 24 hours as described in the text.

individual pens and ad libitum feeding. The rams were split into two groups of eight housed in adjacent rooms. Throughout the study weekly blood samples were collected and other observations made as described in chapter 2.7.

Both groups were initially exposed to 16 weeks of short days (8L:16D) to induce gonadal growth. Group 1 was then exposed to alternating 16 week blocks of long days (16L:8D) and constant dim red light (DD) or constant light (LL), and group 2 was exposed to alternating 16 week blocks of short days and DD or LL. Hourly blood samples were collected for between 49 and 55 hours at the times indicated in fig. 5.1 by the letters A to L. Twenty-seven hours into the sampling periods during the DD and LL treatments (ie. at C,E,G,J) a one hour pulse of either light or dark was given, and this was repeated once every 24 hours for the next two weeks. Further serial samples were collected from the rams in group 2 at the end of this two week period (ie. at H and K) to determine the effects of the pulses on melatonin rhythms.

A second pulse experiment was carried out (indicated by "PULSE" in figure 5.1) after opposite reproductive states had been induced in the two groups of rams by the manipulation of photoperiod indicated in fig. 5.1. The rams in group 2 were transferred from 16L:8D to 32 hours of constant dim red light (DD), and then received a one hour light pulse every 24 hours for two weeks. The start of this light pulse therefore initially occured 8 hours after the "expected" lights on, and the end of the pulse occurred 7 hours prior to the "expected" lights off. Serial hourly blood samples for melatonin assay were collected for 55 hours over the transfer from 16L:8D to DD, and for a further 50 hours two weeks later. The rams in group one transferred from 8L:16D to

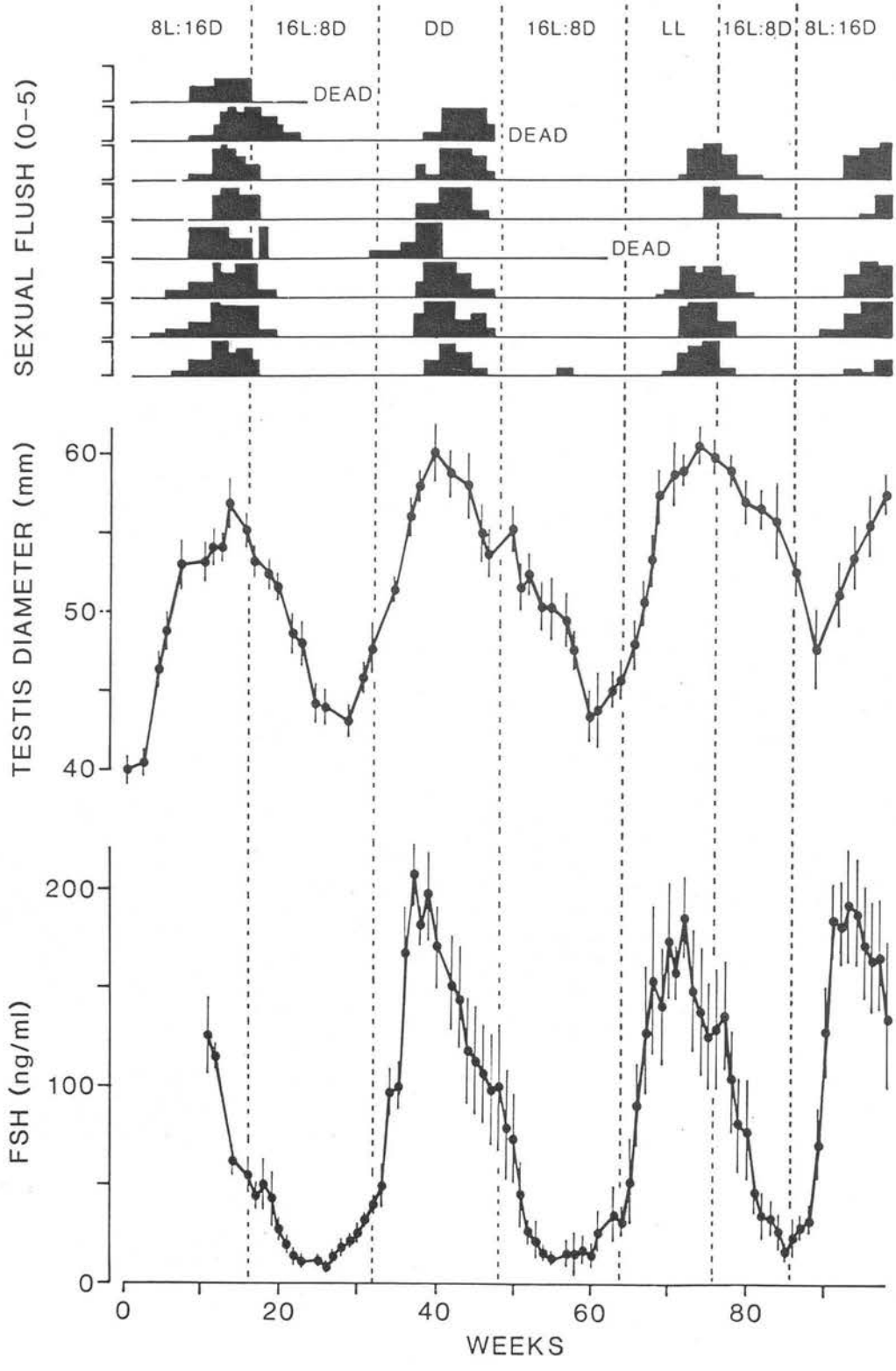

Fig. 5.2

Testicular diameter, plasma FSH levels, and individual sexual flush scores for the rams in group 1. Values are mean ± S.E.M., n=8 at the start of the experiment.

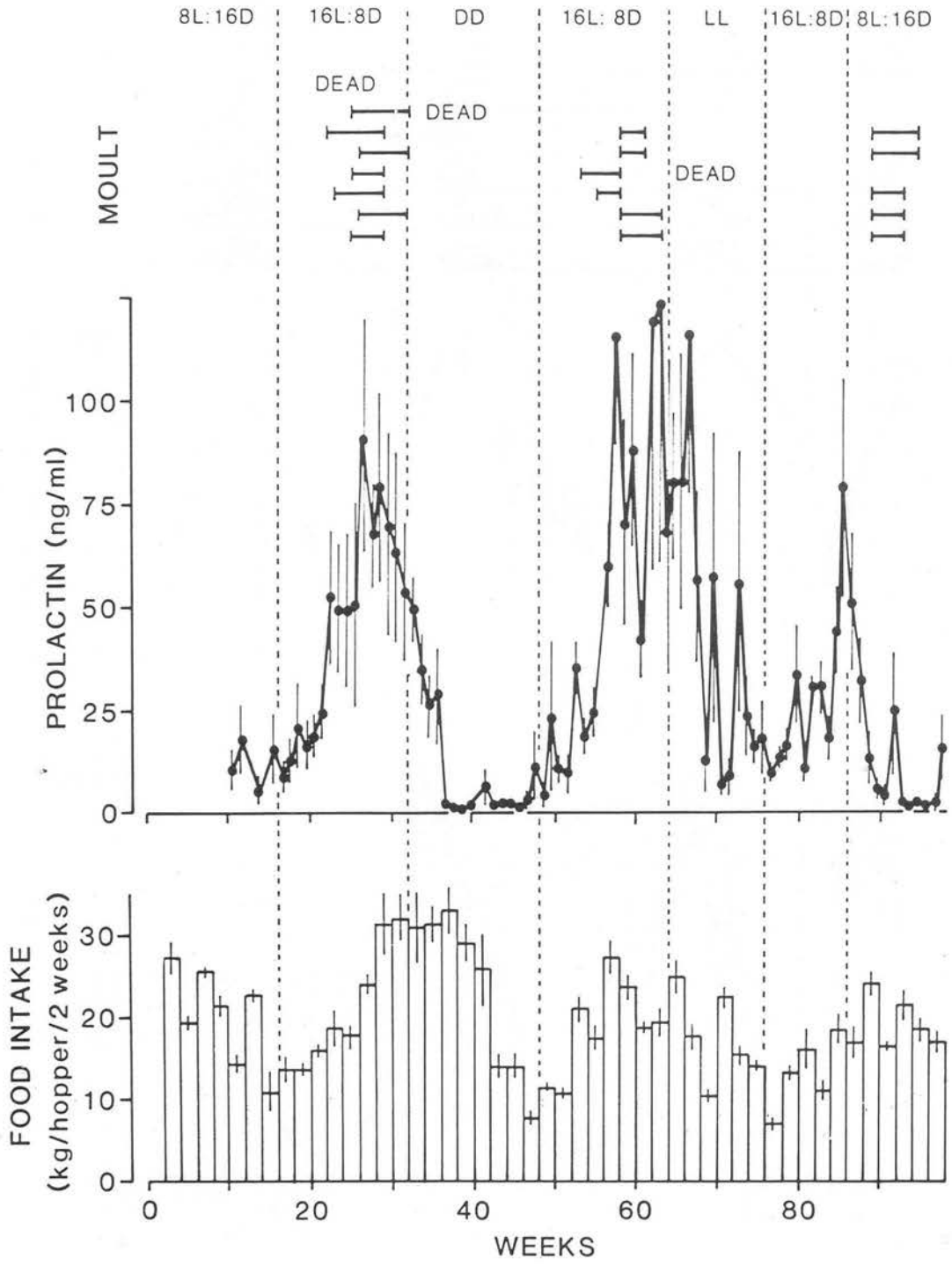

Fig. 5.3

Plasma prolactin levels, food intake, and individual moult duration for the rams in group 1. Values are mean ± S.E.M., n=8 at the start of the experiment.

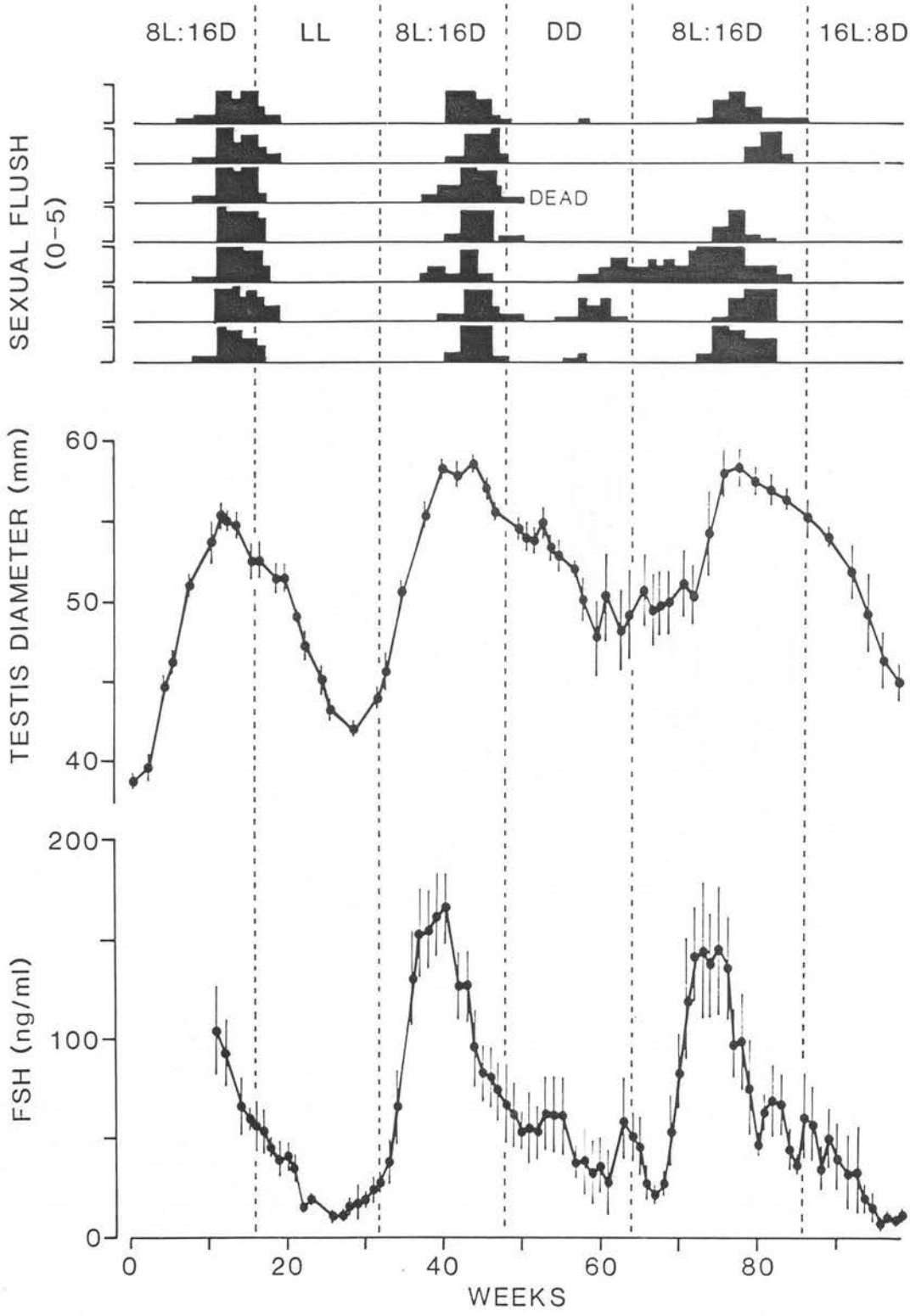

Fig. 5.4

Testicular diameter, plasma FSH levels, and individual sexual flush scores for the rams in group 2. Values are mean ± S.E.M., n=7 at the start of the experiment.

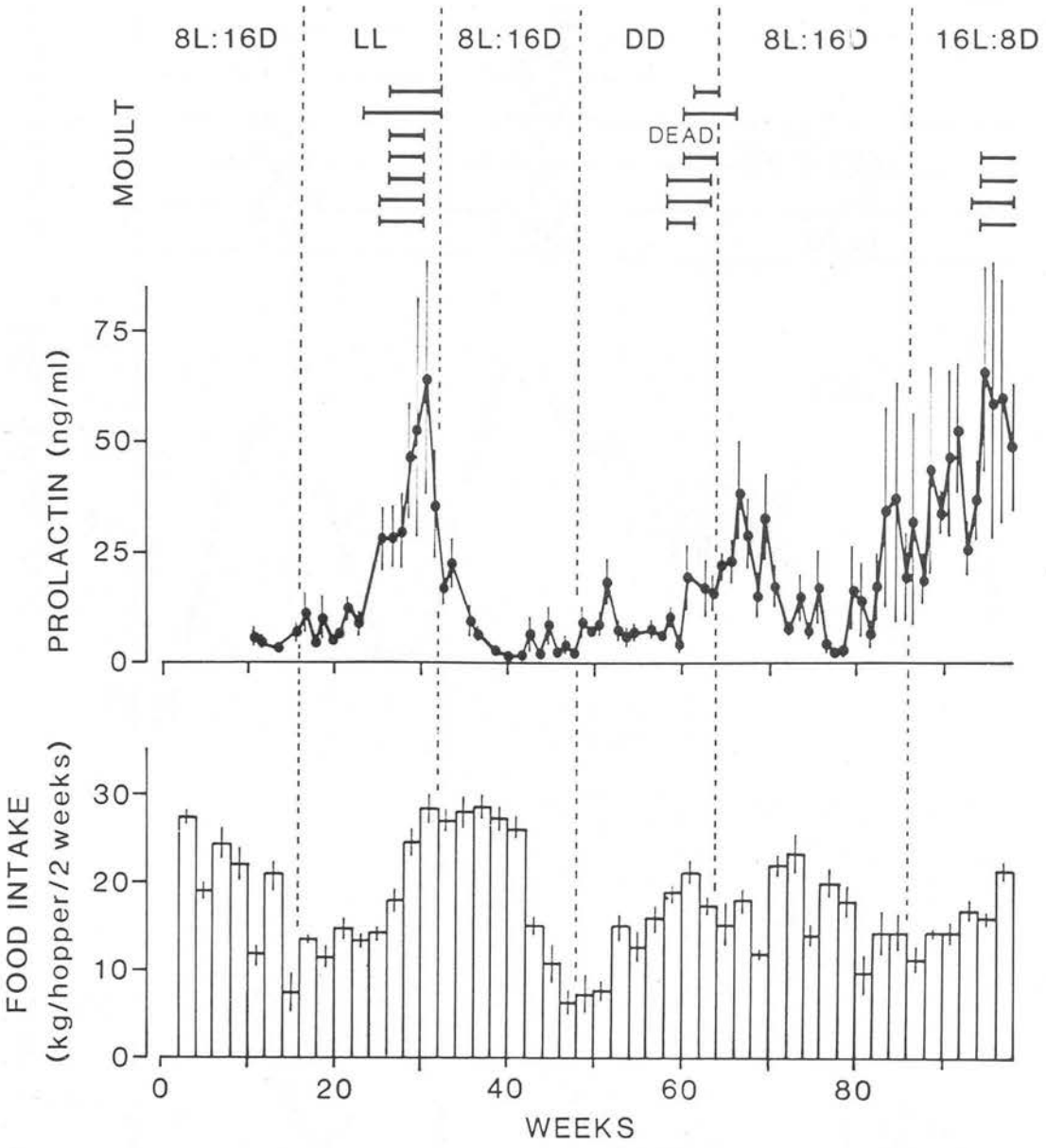

Fig. 5.5

Plasma prolactin levels, food intake, and individual moult duration for the rams in group 2. Values are mean ± S.E.M., n=7 at the start of the experiment.

31 hours of DD, then received two light pulses every 24 hours which were 7 hours apart (1L:7D:1L:15D). The end of the initial light pulse (pulse A in fig. 5.18) thus occurred at the time of the "expected" lights off, and the second pulse (pulse B) occurred midway through the subjective night. Blood samples for melatonin assay were collected as described for group 2. At week 75 the rams which had died during the course of the initial experiment due to urethral calculi were replaced with 1½ year old rams to increase group numbers to the original level for the pulse experiment.

5.3 Results

5.3.1 Effects of constant light and dark on reproductive and somatic cycles

Fig. 5.2 illustrates weekly changes in plasma FSH, testis diameter and sexual flush for the rams in group 1, and fig. 5.3 shows weekly plasma prolactin levels, biweekly food intake, and onset and finish of the moult. Figures 5.4 and 5.5 respectivly show these reproductive and somatic parameters for the rams in group 2.

Statistical analysis of the effects of DD, LL and control photoperiods was carried out using the method adopted by Lincoln and Ebling (1985) which allows comparisons of the rate of change for each parameter. A pretreatment value for each animal was defined as the mean level over the four weeks prece ding the change in photoperiod. The increase or decrease was then calculated as the mean for a 4 week period over which maximal changes have previously been recorded in control experiments where Soay rams have been transferred from long to short photoperiods or *vice versa*. This period was defined as 6-9 weeks post transfer for plasma FSH and prolactin, 9-12 weeks for sexual flush and testis diameter, and 13-16 weeks for food intake. Table 5.6 shows

Table 5.6

Summary of effects of short days, constant dark and constant light on reproductive and somatic parameters

PRE TREATMENT	TREATMENT	TESTIS DIAMETER (mm) PRE	TESTIS DIAMETER (mm) WEEK 9-12	SEXUAL FLUSH (0-5) PRE	SEXUAL FLUSH (0-5) WEEK 9-12	PLASMA FSH (ng/ml) PRE	PLASMA FSH (ng/ml) WEEK 6-9	PLASMA PROLACTIN (ng/ml) PRE	PLASMA PROLACTIN (ng/ml) WEEK 6-9	FOOD INTAKE (kg/animal) PRE	FOOD INTAKE (kg/animal) WEEK 13-16
16L:8D (12 weeks)	8L:16D (control)	43.2 ± 1.6	57.8 ± 1.6	0	3.7 ± 0.2	24.2 ± 10.3	189.2 ± 20.6	42.9 ± 11.3	4.4 ± 1.5	40.8 ± 2.0	24.0 ± 3.6
16L:8D (16 weeks)	DD	45.6 ± 1.1	59.0 ± 1.2	0.1 ± 0.1	3.5 ± 0.3	35.3 ± 6.8	184.7 ± 11.2	66.3 ± 18.9	1.4 ± 0.3	60.6 ± 8.0	20.3 ± 1.5
16L:8D (16 weeks)	LL	43.9 ± 1.4	60.2 ± 1.2	0	3.5 ± 0.16	22.4 ± 5.8	171.9 ± 25.1	84.9 ± 12.4	30.1 ± 11.9	38.4 ± 2.0	20.4 ± 0.8
ANOVAR (pre-treatment)		F=0.84 ns		F=1.01 ns		F=0.81 ns		F=1.67 ns		F=4.1 p<0.05	
ANOVAR (change)		F=0.94 ns		F=1.05 ns		F=0.25 ns		F=0.92 ns		F=4.5 p<0.05	

Values are mean ± S.E.M.

Table 5.7

Summary of effects of long days, constant light and constant dark on reproductive and somatic parameters

PRE TREATMENT	TREATMENT	TESTIS DIAMETER (mm) PRE	TESTIS DIAMETER (mm) WEEK 9-12	SEXUAL FLUSH (0-5) PRE	SEXUAL FLUSH (0-5) WEEK 9-12	PLASMA FSH (ng/ml) PRE	PLASMA FSH (ng/ml) WEEK 6-9	PLASMA PROLACTIN (ng/ml) PRE	PLASMA PROLACTIN (ng/ml) WEEK 6-9	FOOD INTAKE (kg/animal) PRE	FOOD INTAKE (kg/animal) WEEK 13-16
8L:16D (16 weeks)	16L:8D (control)	55.3 ± 0.9	44.1 ± 1.1	3.3 ± 1.0	0	58.6 ± 9.3	10.9 ± 2.7	5.9 ± 1.4	49.4 ± 12.5	33.3 ± 3.2	60.6 ± 8.0
8L:16D (16 weeks)	LL	54.0 ± 0.8	44.2 ± 0.8	3.4 ± 0.2	0	61.6 ± 11.4	17.3 ± 3.17	4.9 ± 0.8	10.3* ± 2.3	27.1 ± 2.0	57.0 ± 2.8
8L:16D (16 weeks)	DD	56.4 ± 0.7	50.0* ± 1.8	1.0 ± 0.4	0.8*** ± 0.4	76.2 ± 14.3	54.3* ± 15.5	3.1 ± 1.3	6.9** ± 1.9	13.7 ± 2.0	36.7 ± 2.6
ANOVAR (pre-treatment)		F=1.95 ns		F=16.2 p<0.001		F=0.65 ns		F=1.35 ns		F=15.4 p<0.001	
ANOVAR (change)		F=4.15 p<0.05		F=20.14 p<0.001		F=3.73 p<0.05		F=6.42 p<0.01		F=0.25 ns	

Values are mean ± S.E.M. Asterisks indicate significantly different changes compared to controls (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). See text for statistical methods.

the mean pretreatment and "maximal" values for rams transferred from long days to short days (the "control" treatment), constant dark or constant light. Table 5.7 shows corresponding values for rams transferred from short days to the various experimental photoperiods. For each parameter both the pretreatment values and the increment over the defined time period have been analysed by one way ANOVAR. Where significant variance occurred individual means were compared with Duncan's new multiple range test.

The data in fig.5.2 clearly show that a rapid rise in plasma FSH occurred in response to transfer from long days to short days, constant dark or constant light, and that there was a corresponding rapid onset of sexual flush and rapid rate of testicular regrowth under all three photoperiods. These observations are supported by the analysis summarised in table 5.6. There were no significant variations in either pretreatment values or in the increments observed. All three photoperiods induced similar rates of decline in plasma prolactin (see fig. 5.3), however the absolute levels of prolactin under LL were considerably higher than those normally observed during rapid testicular development under 8L:16D. ANOVAR of the week 6-9 prolactin levels indicated significantly higher prolactin levels in the LL group compared to the 8L:16D and DD treatments ($F=6.15$, $p<0.025$). A decline in food intake was also observed in response to all three photoperiods. The decrease was significantly greater in the rams transfered to DD, however this was simply due to significantly higher food intake levels prior to treatment.

Transfer of rams from 8L:16D to either 16L:8D (fig.5.2) or LL (fig.5.4) both produced a rapid fall in plasma FSH levels and rapid regression of the testes, whereas transfer to DD produced a much slower

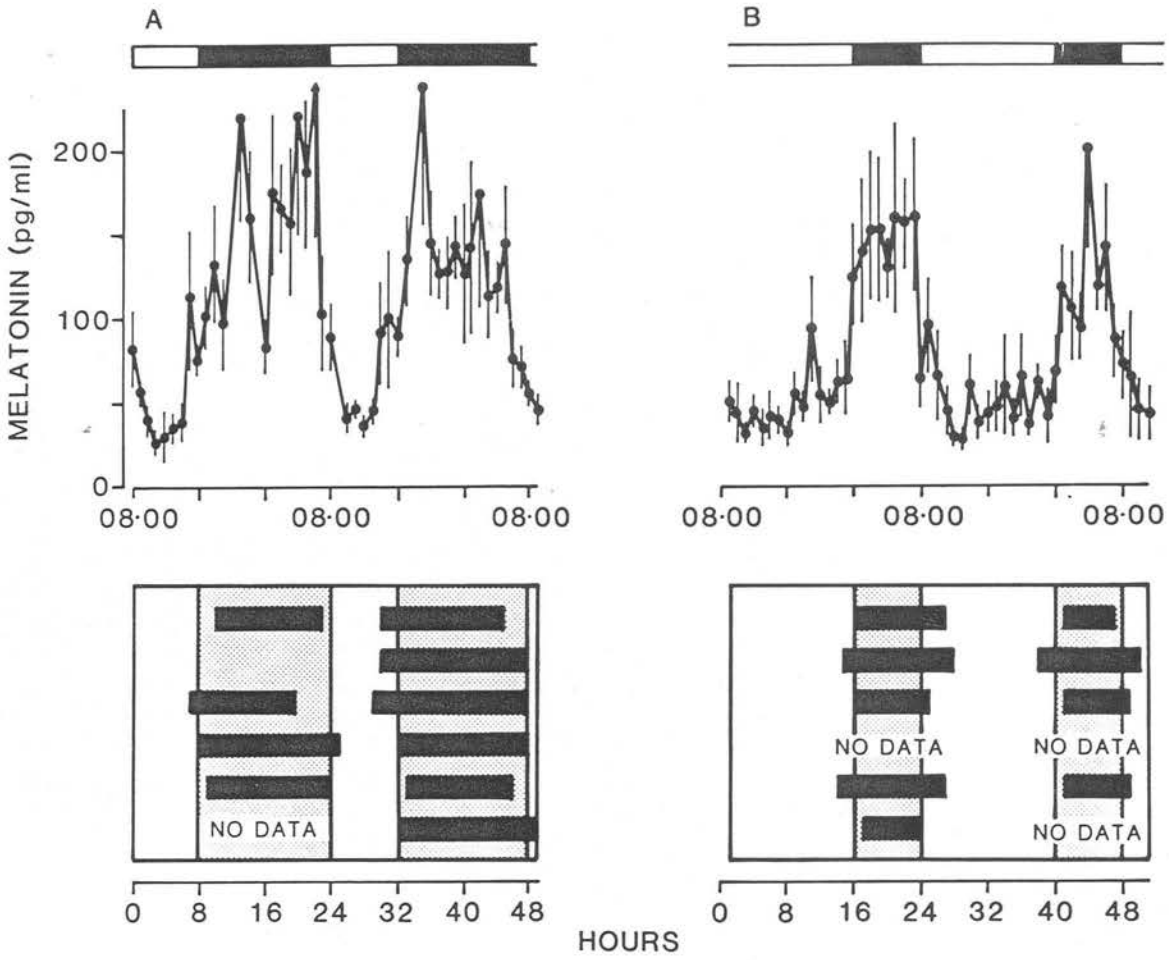

Fig. 5.8

Upper panels show mean plasma melatonin levels in the rams in group 1 sampled after 14 weeks on 8L:16D (A) and after 8 weeks on 16L:8D (B). Values are mean $\pm$ S.E.M. Lower panels correspondingly show significant melatonin peaks in individual rams.

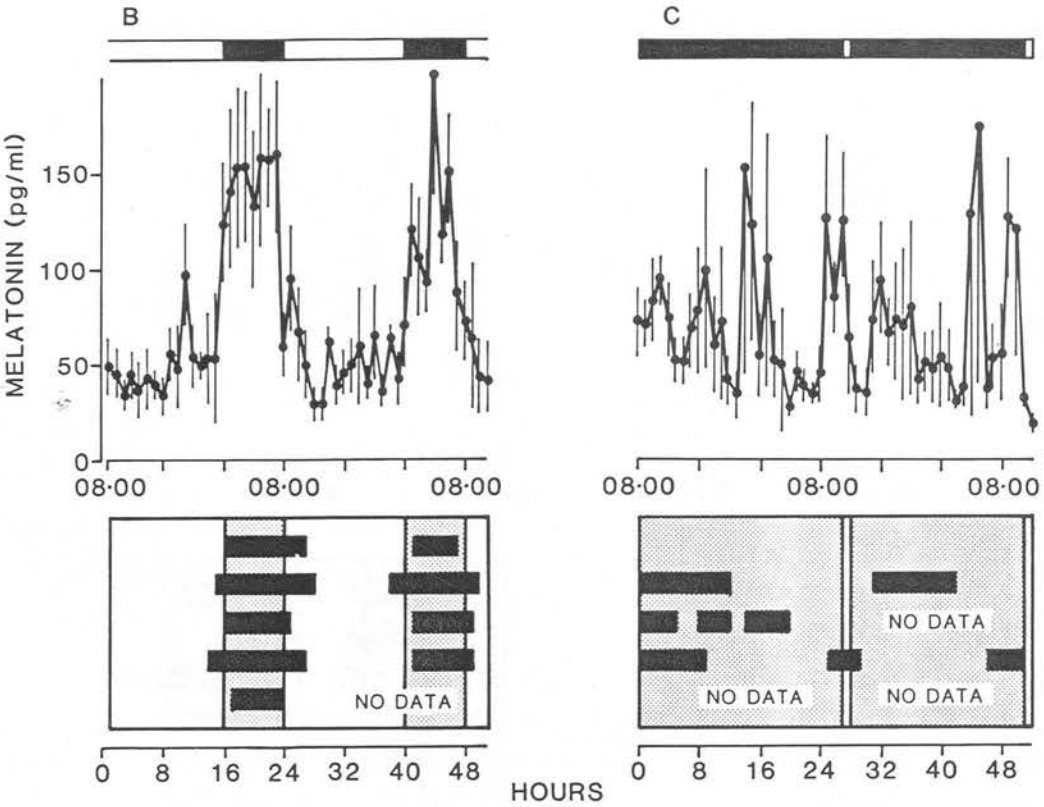

Fig. 5.9

Upper panels show mean plasma melatonin levels in the rams in group 1 sampled on 16L:8D (B) and subsequently after 8 weeks on DD (C). A one hour light pulse was given 27 hours after the start of sampling during (C). Values are mean $\pm$ S.E.M. Lower panels correspondingly show significant melatonin peaks in individual rams.

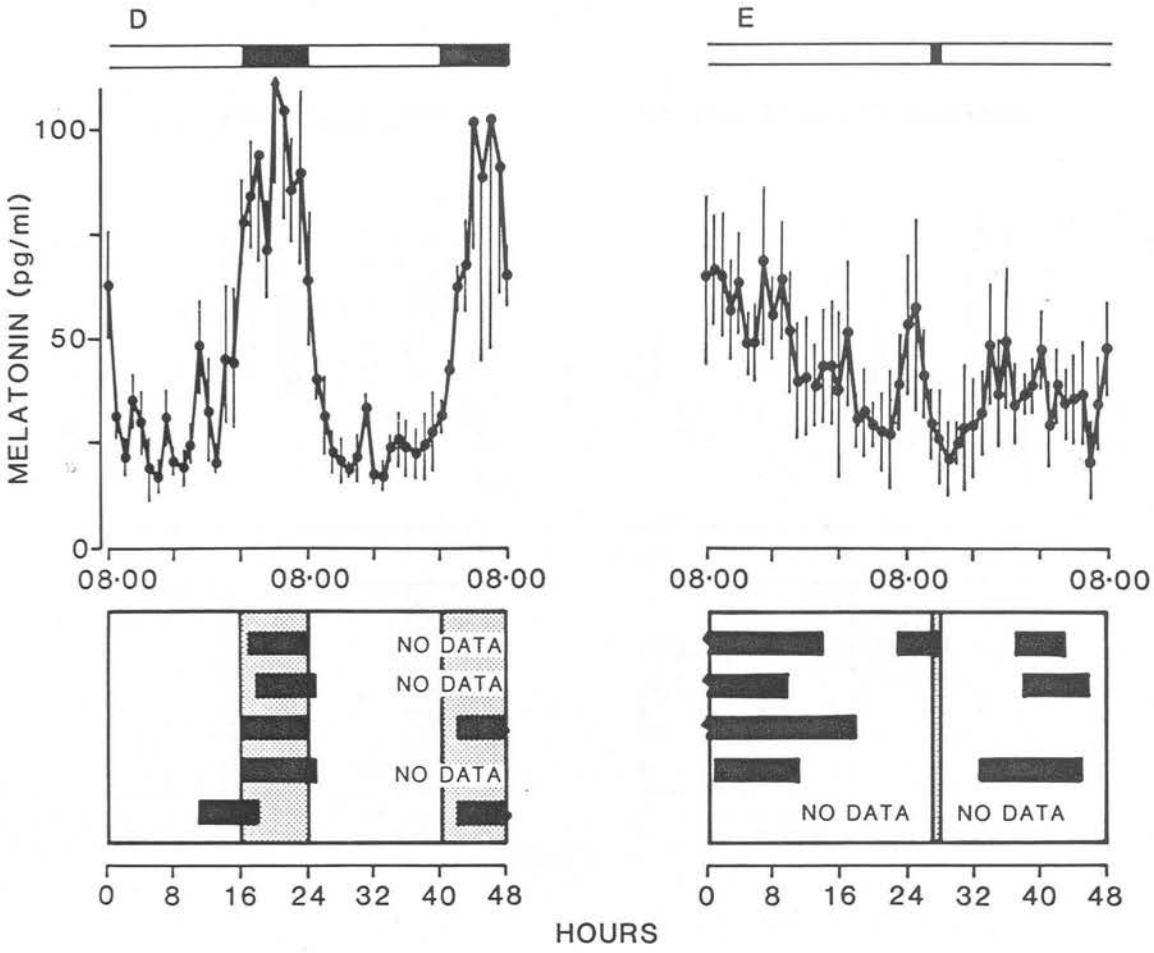

Fig. 5.10

Upper panels show mean plasma melatonin levels in the rams in group 1 sampled on 16L:8D (D) and subsequently after 8 weeks on LL (E). A one hour dark pulse was given 27 hours after the start of sampling during (E). Values are mean $\pm$ S.E.M. Lower panels correspondingly show significant melatonin peaks in individual rams.

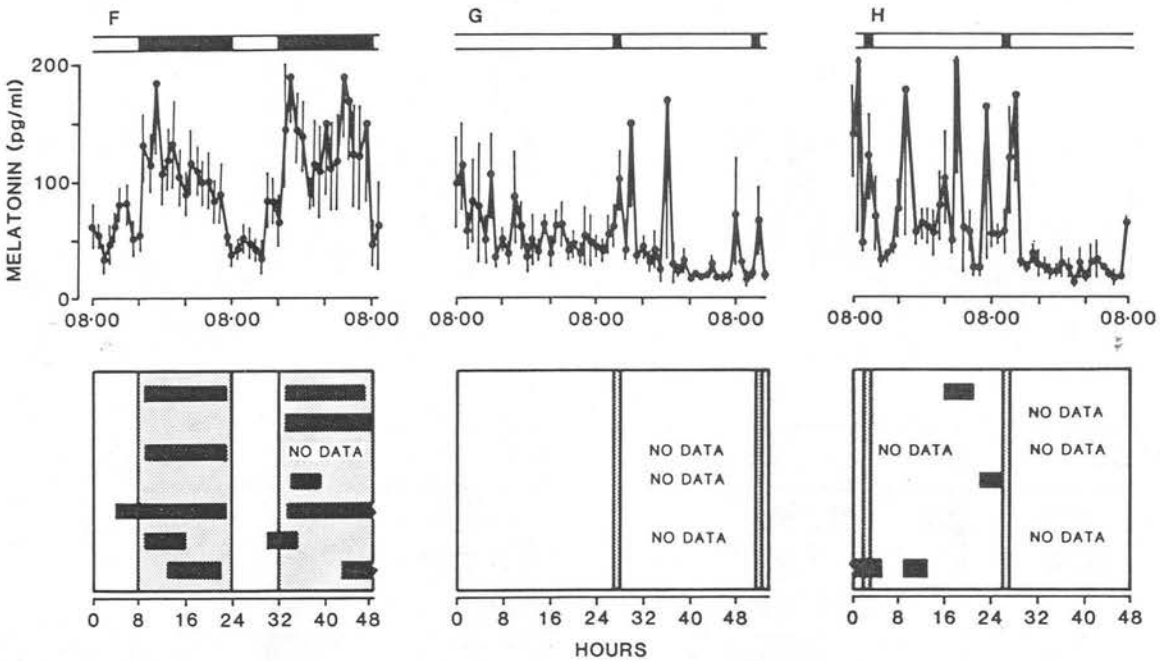

Fig. 5.11

Upper panels show mean plasma melatonin levels in the rams in group 2 on 8L:16D (F) and subsequently after 8 weeks on LL (G). A one hour dark pulse was given 27 hours after the start of sampling during (G), and was repeated every 24 hours. (H) indicates melatonin levels after a further 10 days exposure to 23L:1D. Values are mean ± S.E.M. Lower panels correspondingly show significant melatonin peaks in individual rams.

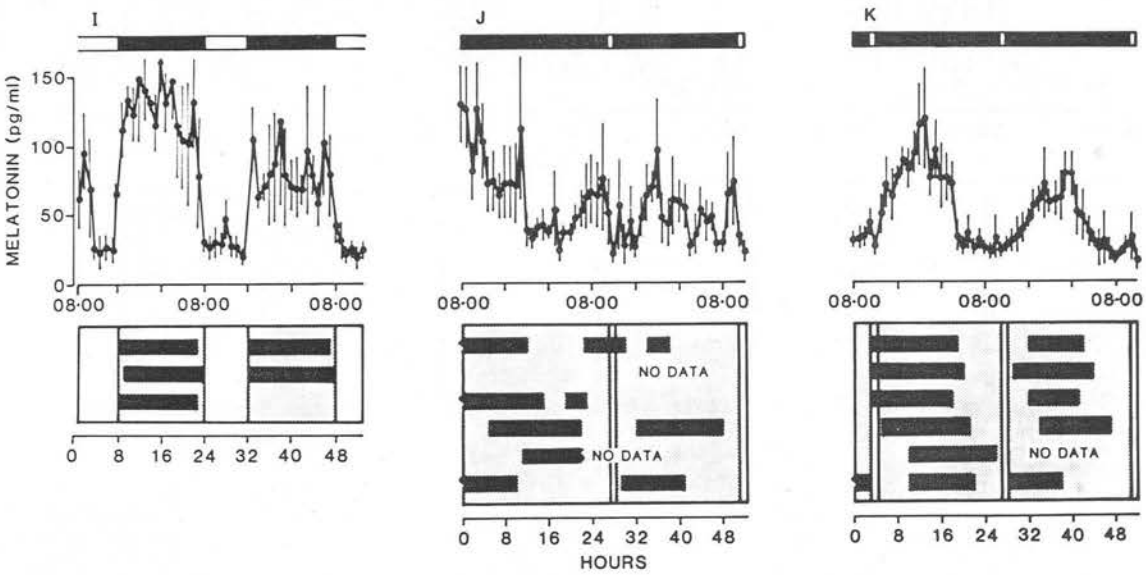

Fig. 5.12

Upper panels show mean plasma melatonin levels in the rams in group 2 on 8L:16D (I) and subsequently after 8 weeks on DD (J). A one hour light pulse was given 27 hours after the start of sampling during (J), and was repeated every 24 hours. (K) indicates melatonin levels after a further 14 days exposure to 1L:23D. Values are mean $\pm$ S.E.M. Lower panels correspondingly show significant melatonin peaks in individual rams.

and more variable decline in FSH levels and testicular activity. Mean values never reached the basal levels observed in controls. The data in table 5.7 indicate that at the nadir of the testicular cycle of the control rams (ie. those on 16L:8D) the decrease in testicular size and plasma FSH were significantly less in the DD treatment group compared to the LL and 16L:8D treated rams. The decline in sexual flush was also significantly less in the DD group even though the pretreatment values in this group were actually lower than in the control and LL groups. The data in figure 5.4 indicate that in 4 out of 6 rams under DD a sexual flush reoccurred prematurely compared to controls. The data in figure 5.5 and table 5.7 indicate that following transfer to both LL and DD the increase in plasma prolactin levels was significantly attenuated compared to the rapid increase observed following transfer from short days to long days. The moult was also slightly delayed in the rams transferred to DD, however no differences in the rate of increase in food intake were observed between the three groups despite the differences in pretreatment values.

5.3.2 Melatonin patterns under DD and LL in relation to photoperiodic responses.

Figures 5.8 -5.12 illustrate the group mean plasma melatonin levels on each of the sampling occasions indicated in figure 5.1 together with the individual melatonin peaks for each ram as defined by the statistical method in chapter 2.9. Fig. 5.8 shows data from two control sampling periods for the group 1 rams; melatonin levels in the long day and short day samples from each ram were measured in the same assay. The subsequent figures show plasma melatonin levels under the experimental photoperiods in relation to melatonin levels in the

Table 5.13

Summary of plasma melatonin parameters under different photoperiods

PRE-TREATMENT	TREATMENT	No. animals with significant peaks (x/n)	mean ONSET relative to lights out (h)	mean DECLINE relative to lights out (h)	FIRST PEAK ONSET relative to start of sampling (08.00) (h)	FIRST PEAK DECLINE relative to start of sampling (08.00) (h)	ALL PEAKS mean DURATION (h)	mean PERIOD (h)
16L:8D	8L:16D	6/6	+0.2 ± 0.54	+15.7 [a] ± 0.56	+8.2 ± 0.54	+23.7 ± 0.56	15.7 [b c] ± 0.7	24.3 ± 1.32
	DD	3/4			0	+9.7 ± 2.03	8.4 [b] ± 2.08	19.2 ± 6.5
	LL	4/4			0	+14.3 ± 1.8	12.4 ± 2.57	27.5 ± 6.6
8L:16D	16L:8D	5/5	0.0 ± 0.45	+9.2 [a] ± 0.58	+16.0 ± 0.45	+25.2 ± 0.58	9.4 [c] ± 0.95	25.0 ± 0.82
	DD	5/6			3.0 ± 2.62	+17.2 ± 2.50	11.6 ± 1.33	21.1 ± 4.50
	LL	0/7			-	-	-	-

Values are mean ± S.E.M.

Values with same subscript differ significantly (a: $p<0.05$, student's t test; b and c: $p<0.05$, ANOVAR)

PERIOD: length of time between the onsets of successive melatonin peaks. This estimate of period and the estimates of period in Tables 5.17 and 5.20 are only approximate because the melatonin rhythms were studied over two 24 hour periods in which the light-dark cycle was not identical.

pretreatment control photoperiod.

Table 5.13 summarises various parameters of the individual melatonin profiles under the different photoperiodic treatments. Values for different treatments have been compared by an ordinary 't' test or one way ANOVAR followed by Duncan's new multiple range test where appropriate. The melatonin rhythms in rams on 8L:16D undergoing rapid testicular recrudescence had a very similar onset of the peak relative to lights off as did those in rams on 16L:8D undergoing testicular regression, however the decline relative to lights off was significantly later in the 8L:16D group, and there was a corresponding significantly increased duration of the melatonin peak. The period of the rhythm in both these groups of animals was very close to 24 hours.

Significant melatonin peaks were also observed in the majority of rams pretreated with long days and subsequently undergoing rapid testicular growth under DD and LL. A large proportion of these peaks were associated with the start of sampling, as illustrated in figures 5.9 and 5.10, thus the estimates of onset and duration are only approximate. In both groups the duration of melatonin peaks was decreased compared to the 8L:16D controls (see table 5.13). There was greater variability between animals in the peak duration, and the periodicity of peaks was extremely variable. The rams kept on DD after a short day pretreatment also showed significant melatonin peaks(see fig. 5.12). These tended to be of a longer duration than those in control rams transferred to 16L:8D, but the periodicity was very variable. As before, high melatonin levels tended to be associated with the start of sampling. None of the rams transferred from 8L:16D to LL showed significant melatonin peaks (see fig. 5.11), though there

Table 5.14

Summary of effects of short light or dark pulses on free running melatonin rhythms.

Sample period (fig 5.1)	J		K			G		H	
DAYS PHOTOPERIOD	0 DD	1 1L:23D	16 1L:23D	17 1L:23D		0 LL	1 23L:1D	9 23L:1D	10 23L:1D
No. animals with signif. peaks (x/n)	5/6	3/4	6/6	5/5		0/7	0/4	3/6	0/4
Relative to END of pulse — ONSET (h)	-	+3.7 ± 2.45	+1.7 ± 1.41	+3.0 ± 1.1		-	0	+12.7 ± 3.8	0
Relative to END of pulse — DECLINE (h)	-	+14.3 ± 3.96	+17.0 ± 1.15	+14.4 ± 1.5		-	0	+17.0 ± 3.79	0
DURATION (h)	13.0 ± 1.3	10.7 ± 3.53	15.5 ± 0.76	11.4 ± 1.12		0	0	4.3 ± 0.33	0
PERIOD (h)	-	-	25.3 ± 2.13			-	-	0	

The analysis of melatonin rhythms suggests that after 8 weeks exposure to LL, melatonin peaks did not persist whereas after 8 weeks exposure to DD significant melatonin peaks occurred in the majority of rams. Exposure to one hour of darkness per 24 hours LL for ten days did not re-establish melatonin peaks whereas exposure to one hour light per 24 hours DD for 17 days had a marked effect on melatonin rhythms. All the rams showed significant melatonin rhythms under the latter lighting regime and there was a synchronisation of the onset of the melatonin peak between rams.

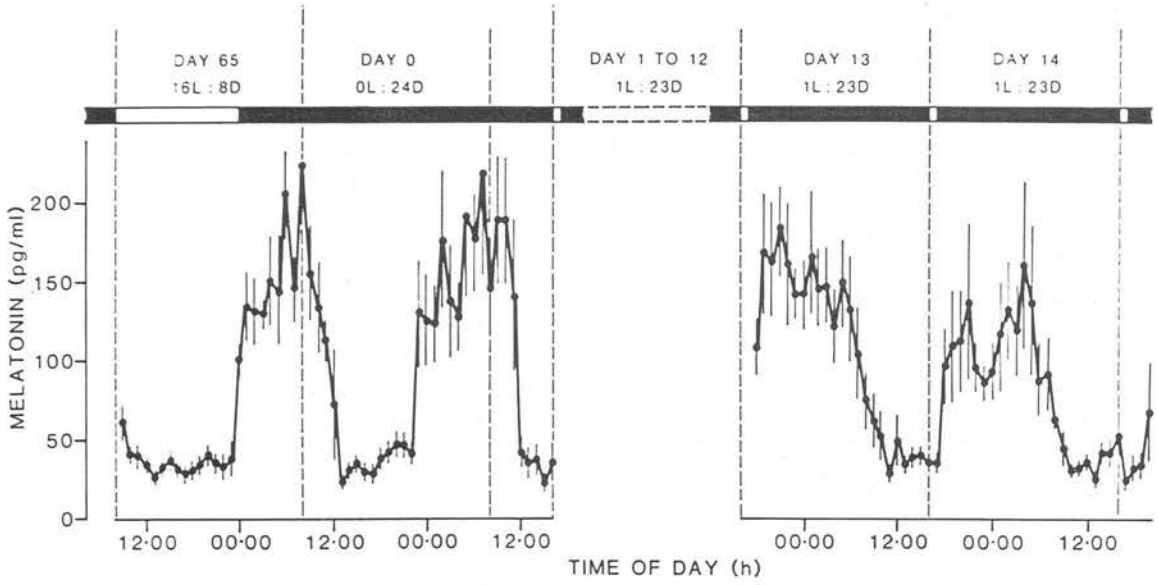

Fig. 5.15

Mean (± S.E.M.) plasma melatonin levels in the rams in group 2 transferred from 16L:8D to constant darkness, and subsequently exposed to single 1 hour light pulse per 24 hours (1L:23D).

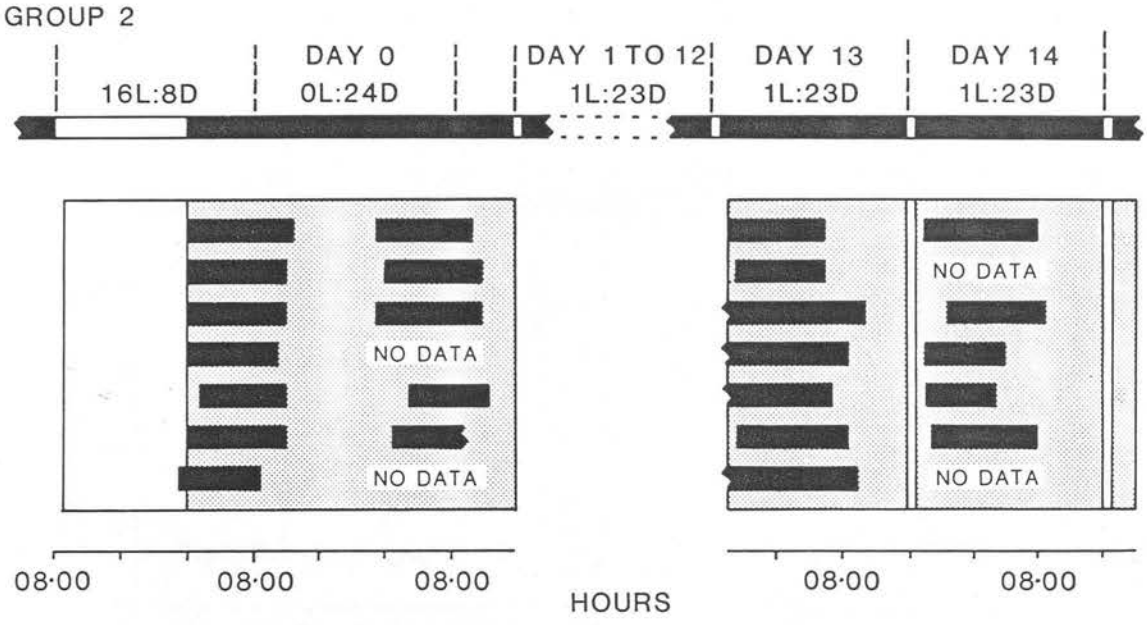

Fig. 5.16

Significant melatonin peaks in individual rams in group 2 transferred from 16L:8D to constant darkness, and subsequently exposed to a single 1 hour light pulse per 24 hours. These correspond with the group mean melatonin levels shown in fig. 5.15.

was no significant difference in absolute melatonin levels compared to the rams transferred from 16L:8D to LL.

5.3.3 Effect of short light or dark pulses on free running melatonin rhythms.

The data in fig. 5.9 and 5.12 (J) indicate that a one hour light pulse given after approximately eight weeks exposure to constant dim red light (DD) had no substantial effect on plasma melatonin rhythms apart from a transitory decrease in melatonin levels at the end of the pulse. However after two weeks exposure to the daily one hour light pulse very clear melatonin rhythms were re-established which were highly synchronised between individual rams as indicated by the clear rhythm seen in the group mean melatonin levels (see fig. 5.12(K)). Table 5.14 summarises various parameters of the melatonin profiles in this experiment and for comparison includes data from the equivalent experiment where daily dark pulses were given to rams previously exposed to constant light for eight weeks. The light pulse given to rams on DD did not significantly alter mean peak duration, but induced all the rams to exhibit melatonin peaks after two weeks with a period close to 24 hours (see fig. 5.12). The dark pulse given to rams on LL had no immediate effect on melatonin levels. After nine days exposure to 23L:1D only half the rams showed significant melatonin peaks. These were of very short duration and were not synchronised between individuals (see fig. 5.11).

5.3.4 Effect of light pulses on established melatonin rhythms.

Figure 5.15 shows the mean melatonin levels for the rams in group two which were transferred from 16L:8D to single one hour light pulse per day, and figure 5.16 illustrates the melatonin peaks in the individual rams. Table 5.17 summarises several parameters of the

Table 5.17

Summary of effects of a single light pulse per day on melatonin rhythms.

		DAY				ANOVAR
		-1	0	13	14	
Photoperiod		16L:8D	DD	1L:23D	1L:23D	
No. animals with signif. peaks (x/n)		7/7	5/5	7/7	5/5	
Relative to expected lights off (00-00)	ONSET (h)	+0.1 ± 0.34	+0.4 ± 0.75	-6.4*** ± 0.38	-5.2*** ± 0.58	F=54.3 p<0.001
	DECLINE (h)	+11.6 ±0.48	+11.6 ±0.51	+8.3** ±0.75	+6.4*** ±1.21	F=15.1 p<0 001
Relative to actual lights off	ONSET (h)	+0.1 ± 0.34		+0.6 ± 0.38	+1.8 ±0.58	F=2.04 ns
	DECLINE (h)	+11.6 ± 0.48		+15.3* ± 0.75	+13.4 ± 1.21	F=4.35 p<0.025
DURATION (h)		11.4 ± 0.43	11.2 ± 0.73	14.7* ± 0.92	11.6 ± 0.93	F=4.29 p<0.025
PERIOD (h)		24.0 ± 0.45		25.4 ± 0.68		t=1.72 ns

Values are mean ± S.E.M. Asterisks indicate values which are significantly different from pre-treatment values (day -1). (* p<0.05, ** p<0.01, *** p<0.001). See text for explanation of statistical methods.

melatonin peaks. The values in each row have been analysed by one way ANOVAR or where appropriate a paired 't' test, and where significant variance due to treatment (time) was observed means were compared using Duncan's new multiple range test.

On the intermediate DD day (day 0) the melatonin rhythms clearly persisted in all animals. The period of the rhythms was 24 hours, thus the onset of the melatonin peak under DD did not phase shift in one day relative to the onset of the peak occuring on the last 16L:8D day. The duration also remained constant, however this duration was longer than that normally observed in rams kept on 16L:8D, indicating that a delayed decline in the melatonin peak had occurred when the subsequent dawn (lights on) was removed. After thirteen days exposure to 1L:23D all the rams showed clear melatonin rhythms which were very closely synchronised as indicated by the clear rhythms in group mean melatonin levels (see fig. 5.15). A very significant phase advance in both the onset and decline of the melatonin peaks occured when these events were expressed relative to real time (the "expected" light off) and compared to the day -1 and day 0 values. When the peak onsets were compared relative to the real lights off (00-00 for day -1 and the end of the light pulse for day 13 and 14) no significant differences were observed (see table 5.17), thus the rams responded to an hour long light pulse as a dusk signal in a similar manner to the way in which they responded to the end of a 16 hour long period of light. There was an increased peak duration on day 13 compared to day -1 and 0 (see table 5.17), however this was probably partly an effect of the start of sampling since the duration on the subsequent day was almost identical to that on days -1 and 0. These elevated melatonin levels at the start

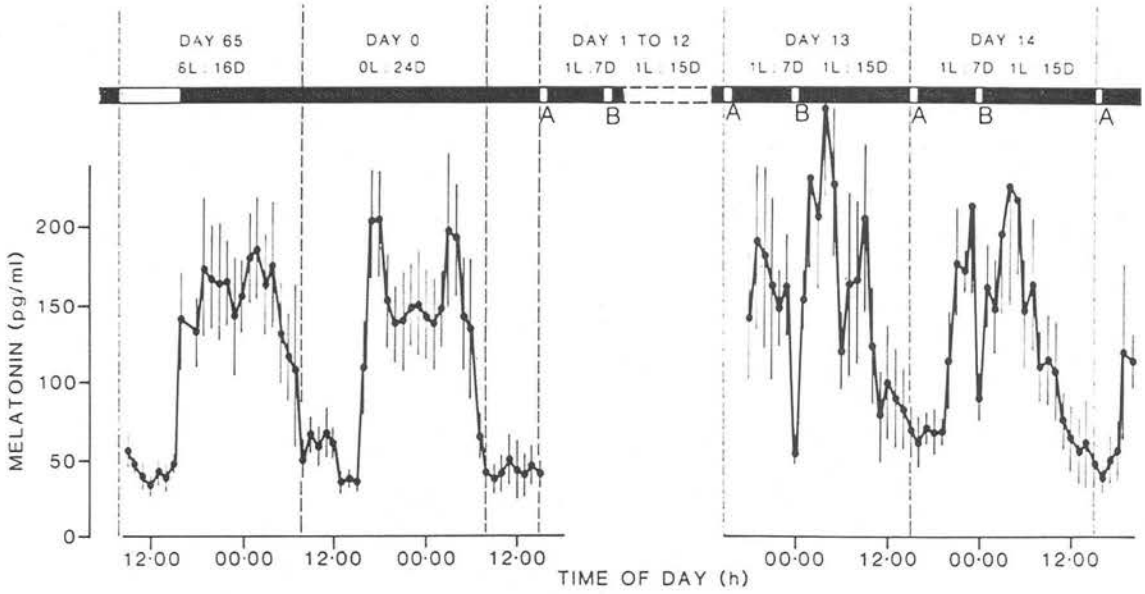

Fig. 5.18

Mean (± S.E.M.) plasma melatonin levels in the rams in group 1 transferred from 8L:16D to constant darkness, and subsequently exposed to two 1 hour light pulses per 24 hours (1L:7D:1L:15D).

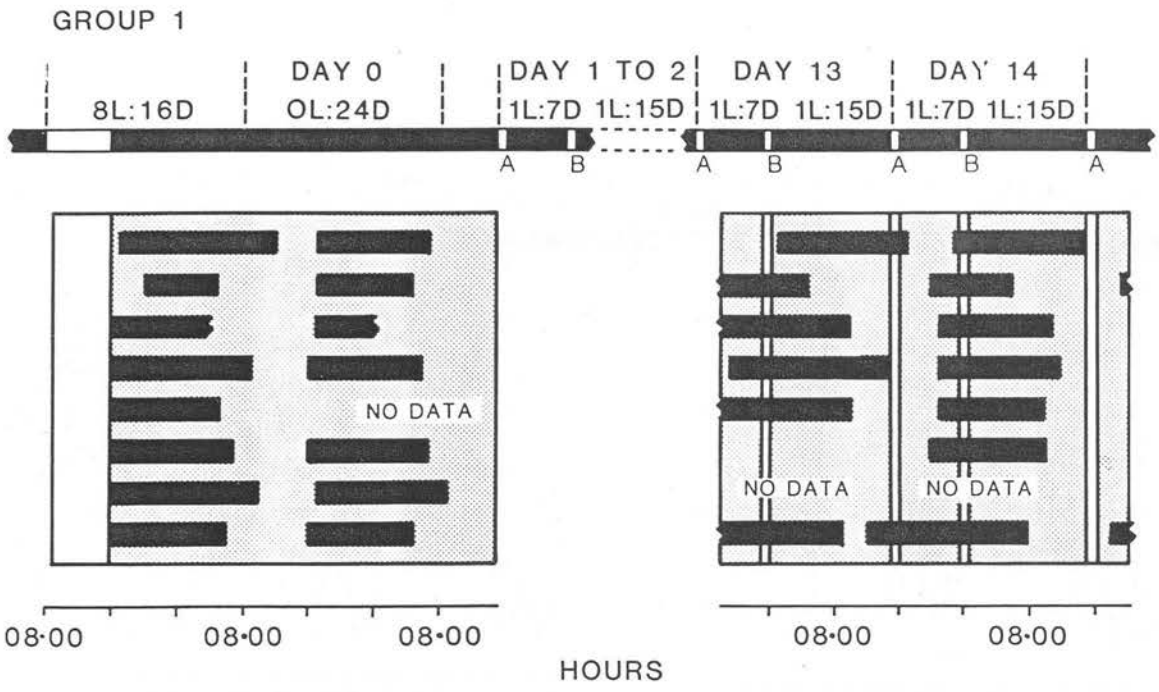

Fig. 5.19

Significant melatonin peaks in individual rams in group 1 transferred from 8L:16D to constant darkness, and subsequently exposed to two 1 hour light pulses per 24 hours. These correspond with the group mean melatonin levels shown in fig. 5.18.

Table 5.20

Summary of effects of two light pulses per day on melatonin rhythms

	DAY -1	DAY 0	DAY 13	DAY 14	ANOVAR
Photoperiod	8L:16D	DD	1L:7D:1L:15D		
No. animals with significant peaks (x/n)	8/8	7/7	6/7	7/7	
Relative to expected lights off — ONSET (h)	0.6 ± 0.50	0.6 ± 0.20	2.7 ± 1.31	3.7 ± 1.34	F=2.91 ns
(16-00) — DECLINE (h)	15.6 ± 0.94	14.5 ± 0.62	19.0 ± 1.77	18.3 ± 1.08	F=3.46 p<0.05
DURATION (h)	14.8 ± 1.15	14.0 ± 0.58	16.7 ± 0.80	14.7 ± 1.15	F=1.18 ns
PERIOD (h)	23.9 ± 0.51		25.0 ± 1.51		t=0.69 ns

Values are mean ± S.E.M. See text for explanation of statistical methods. Onset and decline are expressed relative to the "expected" lights off which also corresponds to the end of the first light pulse (pulse A in fig. 5.18).

of sampling were also reflected in the marginally increased period of the melatonin rhythm under 1L:23D (see table 5.17).

Figures 5.18 and 5.19 show group mean melatonin levels and individual melatonin peaks respectivly for the rams in group 1 which were transferred from 8L:16D to a photoperiodic schedule in which they received two light pulses per day asymmetrically distributed over the 24 hours. The data summarised in table 5.20 were analysed in the same way as for group 2. The short day long duration rhythms free ran into the DD period with a periodicity of 24 hours and an unchanged duration. Unlike the rhythms observed in the rams on long days, the decline of the first peak running into DD was not delayed. After 13 days exposure to 1L:7D:1L:15D melatonin rhythms persisted in all the rams (see Table 5.20). The duration of the peaks and their periodicity were not significantly changed. The data shown in figure 5.18 suggest that there was a large degree of synchrony of melatonin peaks between individual rams. The onset of the peaks relative to real time and real lights off of pulse A was not significantly altered, though there was a trend towards a slightly delayed onset relative to lights off after 13 and 14 days. This effect may have been slightly masked by elevated melatonin levels associated with the start of sampling. The rams did not appear to entrain the onset of their melatonin peaks to the second of the two light pulses (pulse B) which was originally timed to be in the middle of the rams' subjective (ie. "expected") night (see fig. 5.19). This second light pulse did however produce a significant transient decrease in the mean melatonin levels; these returned to normal nocturnal levels within an hour after the end of the light pulse.

Table 5.21

Summary of testicular responses to change in photoperiod and inferred photoperiodic status.

PRE TREATMENT	TREATMENT	RESPONSE	ABILITY TO RESPOND TO SUBSEQUENT CHANGE IN PHOTOPERIOD
16L:8D	8L:16D	Rapid photostimulated development	Rapid regression photosensitive
	DD	Rapid photostimulated development	Rapid regression photosensitive
	LL	Rapid development	Rapid regression photosensitive
	16L:8D [a]	Slow spontaneous development	Slow regression photorefractory
8L:16D	16L:8D	Rapid regression photostimulated	Rapid development photosensitive
	LL	Rapid regression photostimulated	Rapid development photosensitive
	DD	Slow - moderate regression photorefractory	Slow - moderate development photorefractory
	8L:16D [b]	Slow regression photorefractory	Slow development photorefractory

a, b Observations from Almeida and Lincoln (1984a).

5.4 Discussion

Previous studies have demonstrated that in the absence of changing photoperiodic cues cycles of reproductive activity still persist in sheep (see chapter 1.1), thus the responses to manipulations in photoperiod in the initial experiment in this chapter must be interpreted in relation to such spontaneous changes. The overall results of the constant dark and light treatments are summarised in table 5.21 alongside findings from a previous and exactly comparable experiment where Soay rams were maintained for a prolonged period on either 16L:8D or 8L:16D (Almeida, 1982; Almeida and Lincoln, 1984a). The most striking observation in the current experiment was that the rams transferred from 16L:8D to LL showed a rapid switch on of the reproductive axis which was very synchronised between individuals. The original hypothesis was that under LL slow testicular recrudescence would occur as previously observed in rams maintained for a prolonged period on 16L:8D (Almeida, 1982). A second hypothesis was that the rate of regrowth might be slower than that occuring on prolonged 16L:8D because LL would be read by the rams as a "super" long day, thus exposure to LL would break the photorefractoriness which develops to the inhibitory effects of long days (16L:8D). This phenomenon of relative refractoriness has been observed in a few avian species, notably the Japanese Quail (Follett and Robinson, 1980). This species normally undergoes testicular development in response to long days of 12h light per day or longer. Under natural conditions testicular regression occurs in August when the photoperiod is approximately 14L:10D, however at this time quail can be stimulated to regrow their testes by transfer to longer days of 18L:6D, thus photorefractoriness is broken by an increase in the relative photoperiod (Robinson and

Follett, 1982). A third hypothesis was that since the LL treatment was started when the rams were already starting to become refractory to the 16L:8D pre-treatment it might be interpreted as "super" long day which would induce a more rapid photorefractoriness. Although this last hypothesis could account for the observed rapid testicular recrudescence, it is perhaps more likely that the constant light was interpreted as a stimulatory photoperiod since the rams were subsequently fully able to respond to a period of long days (16L:8D), that is, they showed a rapid, synchronised gonadal regression. In the reverse situation where rams were transferred from a stimulatory short day photoperiod to constant light the rams responded in a similar way to control rams which were concurrently transferred from short days to 16L:8D. In both groups rapid testicular regression occured followed by an increase in food intake and the start of the spring moult. Under neither period of LL were the patterns of prolactin secretion the same as those in the rams on control photoperiods. Transfer of rams from 16L:8D to LL induced a decrease in prolactin levels, however mean levels during testicular growth were not as low as the basal levels normally seen in rams undergoing testicular redevelopment on short days. Conversely the increase in prolactin levels in rams transferred from 8L:16D to LL was significantly less than that in rams transferred to 16L:8D. Partial dissociation of the usual inverse relationship between the activity of the prolactin and reproductive axes has been observed under certain other photoperiodic manipulations, for example exposure of ram lambs to short days at 4 months (Howles et al., 1980), exposure of rams to a prolonged period of short days (Almeida and Lincoln, 1984a), in rams receiving a subcutaneous melatonin implant (Lincoln and Ebling, 1985), and in superior cervical ganglionectomized rams housed under

artificial photoperiod (Lincoln, 1979a).

Transfer of rams from 16L:8D to DD induced rapid stimulation of the reproductive axis and a corresponding decline in plasma prolactin levels and food intake, thus DD appeared to be read in this situation as a normal short day. Likewise exposure to DD following 8L:16D pre-treatment resulted in a slow decline in plasma FSH and slow regression of the testes, associated with a gradual increase in plasma prolactin levels and a marginally delayed onset of the moult. This pattern of response was similar to that observed in Soay rams maintained on 8L:16D for a prolonged period (Almeida, 1982), and is indicative of short day photorefractoriness. The variability between rams in the rate of regression when exposed to DD appeared to be greater than that in the previous short day study. Two rams only regressed their testes by about half the normal decrease, and both showed a very premature return of the sexual flush. This could be interpreted as evidence that the rams read DD as a "super" short day which therefore prevented the development of photorefractoriness. However other rams in the group underwent full regression and appeared to be partially able to respond to subsequent exposure to 8L:16D, thus the converse hypothesis that "super" short days induce more rapid photorefractoriness might equally apply. No studies exist in mammals on the relationship between the rate of testicular regression during photorefractoriness and day length, though one study in canaries suggested that photorefractoriness developed more rapidly in birds exposed to an extra long day (20L:4D) than on a normal long day (16L:8D)(Storey and Nicholls, 1976).

Two previous studies have attempted to look at the effects of constant light on sexual activity in sheep. Radford (1960) exposed 2-3

month old merino ewes to LL and observed that the onset of oestrus as indicated by vaginal smears was very similar to that in the controls maintained on the natural photoperiod, though oestrous behaviour was slightly delayed and reduced. Ducker et al. (1973) exposed Clun Forest ewe lambs to LL from birth and also found that onset of oestrous cyclicity was not significantly different from lambs reared on 6L:18D, 12L:12D or 18L:6D. These studies are not directly comparable with the present study for several reasons. Firstly both studies used pre-pubertal animals, those in the latter study had no long day pre-treatment which appears to be prerequisite in setting photoperiod responses in lambs (Foster, 1983). Both studies used raddled rams to detect oestrus; in weakly photoperiodic breeds such as the merino social influences such as the "ram effect" have a marked influence on sexual cyclicity (Martin, 1984). Thirdly, in Radford's study LL was provided by keeping ewes in daylight and supplementing light at night with fluorescent tubes, thus the ewes would still have experienced a large diurnal fluctuation in light intensity. Despite these differences, both these studies concur with the concurrent observations that LL per se is not inhibitory to the reproductive axis in sheep, and indicate the importance of underlying spontaneous cycles of reproductive activity in the absence of photoperiodic cues.

An important question is whether the observed photoperiodic responses can be explained in terms of the observed melatonin rhythms. Under both 8L:16D and 16L:8D the period of elevated melatonin secretion (the "peak") approximately mirrored the dark phase. Since both the duration and the temporal relationship of the onset and decline of the melatonin peak differed significantly between stimulatory short days and inhibitory long days these observations equally support the

duration and internal coincidence hypothesis outlined in chapter 1.1.4. The data in table 5.13 indicate that the rapid testicular growth which occurred under LL was associated with significant melatonin peaks of 12 hours mean duration, whereas no peaks were detectable when testicular regression was occuring under LL. Significant melatonin peaks were also detectable when rapid testicular growth occured under DD though the mean duration was only 8.4 hours. This does not easily support a duration hypothesis, however inspection of the individual data in figures 5.9 - 5.12 (C,E,J) shows that rarely under DD and LL did the melatonin profiles show a clear circadian periodicity, thus the data poorly support an alternative internal coincidence hypothesis. The data are limited by small group sizes and technical problems of collecting serial blood samples from rams for prolonged periods in darkness. Furthermore, the data are distorted by the high incidence of peaks occuring at the start of sampling. This is reflected in the group mean melatonin values as a gradual decrease in levels over the 50 hours sampling periods under DD and LL, and may indicate a non-specific stress effect enhancing melatonin secretion at the start of a sampling period. Such effects may have been overlooked in previous studies of melatonin rhythms under well defined short, long and natural photoperiods where the clear diurnal variation in melatonin levels might override the stress effect and where sampling in many studies has been started in the morning coincident with lights on thus a few high melatonin values might be interpreted as a slow decline in the previous nocturnal levels. It is unlikely that the elevated levels at the start of sampling under constant photoperiods represented the effects of external zeitgebers such as temperature since they were not repeated in the second sampling day, and did not change with external season. The

"stress" effect was also observed in the last pulse experiment when sampling was started in the late afternoon.

One possible explanation for the non-circadian periodicity and variable duration of melatonin peaks in a given sampling period is that under prolonged LL and DD there is a breakdown in the internal synchrony of multiple oscillators which might govern the secretion of melatonin. Evidence for such a desynchronisation has been obtained in Syrian hamsters and in rats in which "split rhythms" of locomotor activity have been recorded during long term exposure to continuous illumination (Pickard and Turek, 1982;Turek et al., 1982; Cheung and McCormack, 1983). Dissociation of body rhythms has also been observed in human subjects kept in isolated bunkers, indicating desynchronisation of endogenous oscillators which govern them (Aschoff, 1981). It is suggested that two oscillators govern pineal N-acetyl transferase activity in the rat on the basis of their different entrainment properties by light pulses (Illnerova and Vanecek, 1982), thus dissociation of melatonin rhythms under DD and LL might be predicted. To demonstrate this adequately it would be necessary to study melatonin levels over the course of many days.

It is difficult to interpret the significance of melatonin rhythms under DD following 8L:16D pre-treatment when the rams showed typical photorefractory responses. Certain studies have demonstrated a loss of melatonin rhythms during refractoriness and thus postulated that photorefractoriness is an inability of the melatonin rhythms generating system to convey photoperiodic information (Almeida and Lincoln, 1984a). Other studies however show no disturbance of melatonin rhythms during refractoriness, for example rhythms in Syrian hamster pineal melatonin content are normal during refractoriness to short days (Rollag et al.,

1980). Additionally many studies indicate that exogenous melatonin treatments are unable to affect short term responses when given to photorefractory animals (Bittman, 1978; Turek and Losee, 1978; Lincoln and Ebling, 1985), thus supporting the hypothesis that photorefractoriness results from the inability of the hypothalamus to continue to respond to the pineal melatonin signal. Since the melatonin rhythms observed under DD were of variable periodicity and duration it is not surprising that photorefractory responses were observed. The mean duration of melatonin peaks was infact less than that normally observed under 8L:16D; it is perhaps suprising that a true long day response was not observed, that is, a synchronised rapid testicular regression and rapid increase in prolactin secretion. A recent study indicates that 8 hour long infusions of melatonin given to pinealectomized ewes induce a long term response in the reproductive axis as evidenced by an increase in the negative feedback potency of oestradiol implants (Bittman and Karsch, 1984). This study did not further investigate whether other aspects of a long day photoperiodic response occurred, for example elevated prolactin secretion, thus it is not fully established that short durations _per se_ are all that is necessary to mimic all aspects of a long day photoperiod.

It is evident that the short term effects of transferring rams from 16L:8D to LL were different to the effects of pinealectomy or superior cervical ganglionectomy and subsequent exposure to 8L;16D. Constant illumination with fluorescent light did not suppress melatonin levels whereas levels in ganglionectomized or pinealectomized sheep are low or undetectable (Lincoln _et al._, 1981, 1982; Kennaway _et al._, 1977; Lincoln and Cunningham, unpublished observations). Using a different RIA, Kennaway _et al._ (1983) have reported that melatonin levels in ewes kept on LL are consistently less than 18pg/ml. It seems possible in the current study that it was some aspect of melatonin secretion under LL that induced

rapid testicular growth even though the experimental design could not unequivocally demonstrate this.

This study also suggests that LL is not an identical treatment to DD. One hour dark pulses given to rams on LL had no clear effects on plasma melatonin levels, either in terms of a direct response, or after repeated exposure. One hour light pulses given to rams on DD with previously poorly defined and variable melatonin rhythms had a very significant effect when given repeatedly once per 24 hours for two weeks. By day 15 of the light pulse treatment all the rams showed significant melatonin peaks with a periodicity close to 24 hours. The peaks were relativly synchronised between individuals, and the onset was shortly after the light pulse which suggests that the primary effect of the light pulse is to act as a "dusk" entraining signal rather than to produce direct suppression of melatonin secretion.

The second pulse experiment supports this finding. Rams which had a precise melatonin rhythm free running in DD received a one hour light pulse which finished 7 hours earlier than the "expected dusk", that is, 7 hours before the onset of the existing melatonin peak at the start of the pulse treatment. After thirteen days the melatonin peaks were synchronised to start shortly after this light pulse finished, thus it appears that over the course of the treatment the melatonin rhythms had been phase advanced by several hours. Interestingly the duration did not change markedly and was similar to that previously observed in the first 1L:23D experiment. When the rams were first transferred from 16L:8D to DD it was noticeable that the duration of the melatonin peak immediately increased from the expected 8 hours to 11.4 hours, presumably because the influence of the dawn lights on was absent. The other group of rams transferred from 8L:16D did not show an

increased duration when transferred to DD. It is therefore possible that either the SCN cannot stimulate the pineal for longer than 16 hours, or the pineal gland itself is unable to secrete melatonin for longer than this. In the presence of inadequate photoperiodic cues a free running rhythm of melatonin secretion is observed, but its duration cannot therefore exceed 16 hours. This hypothesis would explain why none of the rams maintained on DD had continuously high melatonin levels.

Based on this assumption a simple hypothesis can be constructed to explain the generation of melatonin rhythms. In sheep dusk entrains the onset of the melatonin peak. The "endogenous" duration of this peak is 16 hours - a short day duration - thus the direct effect of light is to suppress melatonin secretion at dawn. This sets the actual duration of the melatonin peak and therefore conveys photoperiodic information ultimately to the hypothalamus. A similar hypothesis has been proposed to explain the generation of rhythms in pineal melatonin content in the Syrian hamster, though the entrainment and direct effects are reversed (Tamarkin et al., 1980a). Their study demonstrated that the rapid morning decrease in pineal melatonin content in hamsters could not be immediately altered by extending the dark phase, whereas the evening rise could be delayed by extending the light period, thus the decline is the entrained endogenous event, and the onset is directly set by the onset of darkness.

The aim of the second part of the pulse experiment was to test the proposed hypothesis. The results clearly demonstrate that the first pulse (pulse A in figure 5.18) provided the dusk entrainment cue. The second pulse (pulse B in figure 5.18) only produced a transient effect on melatonin levels. It is possible that this second pulse was not of

sufficient length to suppress melatonin levels for the rest of the night, however this seems unlikely since in rodents night interruption pulses from 15 minutes to less than one second in duration can produce profound photoperiodic responses (Hoffman and Melvin, 1974; Hoffmann, 1982; Earnest and Turek, 1983; Ellis and Follett, 1983). These responses are presumably induced by altered pineal melatonin secretion since pinealectomy can block the stimulatory response to skeleton long days (Rudeen and Reiter, 1980). A more satisfactory model to account for the current data is to hypothesise that both the onset and decline of the melatonin peak are determined by endogenous oscillators which entrain to the environmental light dark cycle. The interaction of multiple endogenous oscillators in generating circadian rhythms has been extensivly discussed by Pittendrigh (1981). In the current study the effectiveness of the second pulse of light in allowing pineal melatonin secretion might therefore depend on its temporal relationship to these endogenous oscillators rather than its direct effect on melatonin secretion. Studies on photoperiodic responses to skeleton photoperiods provide evidence for this model. In rams maintained on short days but given a one hour light pulse at different times in the dark phase a photoinducible phase for the stimulation of prolactin secretion has been demonstrated (Ravault and Ortavant, 1977; Ravault et al., 1981). Plasma melatonin levels were not measured in these studies, however a recent study indicates that pinealectomy blocks the long day prolactin response to a skeleton photoperiod (Brinklow and Forbes, 1984), thus it seems likely that the effects of skeleton photoperiods on prolactin secretion are mediated through changes in pineal melatonin secretion. The failure of pulse B to convincingly suppress melatonin levels in the current study would therefore be

because it occurs too early in the subjective might to provide a stable entrainment signal. Some direct support for this hypothesis has recently been obtained by Brinklow et al., 1984. Melatonin rhythms in lambs growing on a skeleton photoperiod of 7L:10D:1L:6D were compared to those in lambs reared on 8L:16D. Following the light pulse which occured "late" in the subjective night, melatonin levels were suppressed for the remainder of the dark phase. The resulting melatonin profiles were therefore of shorter duration which would explain the long-day stimulation of prolactin release which was observed. The studies on the different temporal effects of light discussed in chapter 1.1.3 generally support the two oscillator model, and direct evidence is provided by the studies of Illnerova and Vanecek (1982) demonstrating that the separate oscillators which control the onset and decline in pineal N-acetyl trasferase activity in rats have separate phase response curves.

A corollary of the two oscillator model is that the supposedly separate direct and entrainment effects of light might be qualitatively the same phenomenon. A direct effect of light could be considered as one where entrainment is instantaneous. It would be interesting to observe whether the bright light suppression of melatonin levels in humans reported by Lewy et al. (1980) altered melatonin rhythms on subsequent days. Continuous illumination provides no entrainment cues, though may mask the expression of the underlying free running rhythm, thus when Perlow et al. (1981) exposed Rhesus monkeys to constant light melatonin levels were suppressed, but re-emerged following transfer to DD in a similar phase relationship to that under the 12L:12D photoperiod to which the monkeys had been initially exposed.

A final point of interest in the current study is that although the second light pulse (B) was not temporally related to the melatonin rhythm entrained by pulse A in such a way as to produce a normal decline in melatonin secretion, none of the rams appeared to reverse the photoperiod such that the onset of the melatonin rhythm would be entrained to pulse B and the endogenous duration of melatonin secretion would occur in the longer 15 hour period of darkness. Studies with several avian species indicate that when night interruption experiments are carried out with light pulses late in the subjective night the birds instantly reverse the photoperiod to read the light pulse as dawn, thus the main block of light falls in the early part of the new subjective night (Follett, 1973, 1981; Follett and Milette, 1982). Since measurement of daylength in birds does not appear to involve a pineal melatonin mechanism (Turek and Wolfson, 1978; Simpson et al., 1983) it is interesting to speculate whether this difference in entrainment responses between birds and sheep is a reflection of the conservatism of the melatonin mediated photoperiodic response in sheep compared to the rapid photoperiodic responses observed in birds.

5.5 Summary

1) Refractoriness which develops in Soay rams after prolonged exposure to short days (8L:16D) cannot be prevented by increasing the length of the dark phase to constant dark (0L:24D), that is, Soay rams do not show relative photorefractoriness.

2) Exposure of rams which are becoming photorefractory to the inhibitory effects of long days to constant light results in rapid gonadal recrudescence. This rapid development has not been previously

observed in Soay rams in which the pineal melatonin rhythm generating system has been ablated. It is therefore possible that some aspect of melatonin secretion under LL is inducing or permitting the apparant "short day" response.

3) After eight weeks exposure to constant dark, the plasma melatonin rhythms in Soay rams are disorganised. A one hour light pulse per 24 hours is sufficient to reorganise the melatonin rhythm; the onset of the peak being shortly after the end of the light pulse.

4) Melatonin rhythms in rams maintained on both long days (ie. short duration peaks) and short days (ie. long duration peaks) will free run when the rams are transferred to constant dark. The duration of the peak in the "long day" rhythms increases whereas the duration of the peak in the "short day" rhythm remains constant.

5) A one hour light pulse given several hours before the onset of the free-running melatonin peak phase advances the melatonin peak. After two weeks repeated exposure the onset of the melatonin peak is shortly after the light pulse, thus the pulse appears to provide a "dusk" entrainment cue. A second pulse given eight hours after the first only induces a transient decrease in melatonin levels.

Chapter 6

General discussion

The endogenous basis of melatonin rhythms in mammals is now well established. In attempting to understand photoperiodic responses the question of how the melatonin rhythm is influenced by the enviromental light-dark cycle is of considerable importance. Constant light does not constantly suppress melatonin levels in Soay rams, and conversely melatonin levels are not constantly elevated to normal nocturnal levels in rams kept on constant dark. It seems highly unlikely that under natural light dark cycles direct suppression by light and direct stimulation by darkness is responsible for generating the observed melatonin rhythm. Very clear free running melatonin rhythms were observed in rams transferred from both short days and long days to constant dark. These free running rhythms can be entrained by a one hour light pulse given once per 24 hours. In this type of skeleton photoperiod experiment the light pulse appears to be interpreted as a dusk signal because onset of the melatonin peak occurs within a few hours of the end of the pulse. A second one hour light pulse given seven hours after the end of the first only produces a transient suppression of melatonin levels. These results in combination with data from other skeleton photoperiod experiments in mammals suggest that the effectiveness of light pulses in altering melatonin levels depends not only on their intensity, but more importantly on their temporal relationship to the underlying melatonin rhythm, thus the primary action of light pulses in skeleton photoperiod experiments is as entrainment cues. These data are consistent with the hypothesis that generation of melatonin rhythms is dependant on the internal coincidence of two groups of endogenous oscillators (Illnerova and Vanecek, 1982). The importance of circadian rhythms in photoperiodic time measurement is therefore in the generation of melatonin rhythms.

Perhaps the "photoinducible phase" first proposed by Bünning in 1936 (see reviews by Follett, 1973; Elliot, 1976) represents a period when a light pulse is able to entrain the dawn oscillator such that the duration of the melatonin peak is shortened. This hypothesis has not been tested in rodents because despite several elegant skeleton and resonance photoperiod studies it has not proved technically feasible to measure plasma melatonin rhythms. A resonance experiment in sheep does provide evidence that circadian rhythms are necessary to entrain melatonin rhythms in a stable manner. A photoperiod with periodicity of 36 hours (8L:28D) induced disrupted melatonin rhythms, thus rapid testicular development was prevented, whereas a 8L:40D photoperiod induced long duration melatonin rhythms with a 24 hour periodicity and hence rapid gonadal growth (Almeida and Lincoln, 1982).

The current studies are consistent with the view that the eventual interpretation of the melatonin rhythms as a daylength signal is determined by the duration of the melatonin peak. Two elegant series of experiments where infusions of melatonin have been given to pinealectomized sheep (Bittman et al., 1983b; Bittman and Karsch, 1984) and Djungarian hamsters (Carter et al., 1983a,b;Goldman et al., 1984) provide good evidence for the duration hypothesis. However, they are not yet adequate to reject the possibility that interpretation of melatonin rhythms also requires a circadian mechanism because the duration protocols always delivered melatonin in periods with a 24 hour periodicity. If melatonin or other zeitgebers were also entraining some form of circadian rhythm in melatonin sensitivity it is possible that the difference between the responses to long and short melatonin durations are due to the difference in onset and decline of the peak consistently falling on a different part of the phase response curve of

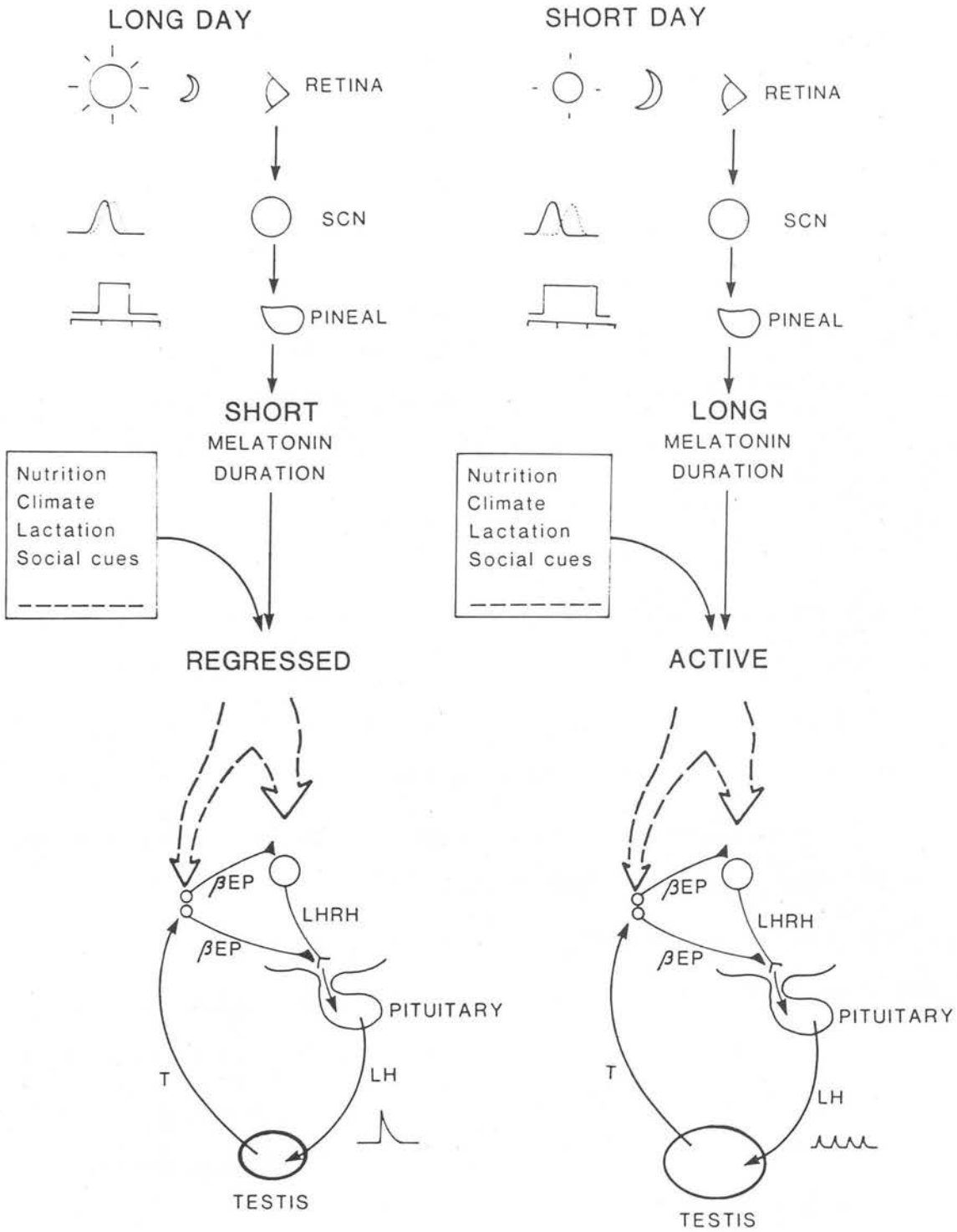

Fig. 6.1

Control of seasonal breeding in the Soay ram. This model encompasses the observations on melatonin rhythm generation and those on the role of EOP in the control of LHRH release. The environmental light-dark cycle entrains endogenous circadian oscillators, thus producing a long or short melatonin duration signal to the hypothalamus. This photoperiodic information along with other neural inputs ultimately drives the neurosecretion of LHRH. The hypothalamic-pituitary-gonadal axis is seen as a homeostatic system in which EOP are involved in relaying inhibitory effects of testosterone on stimulatory inputs to the LHRH pulse generator and on release of LHRH from terminals in the median eminence. The actions of photoperiod are therefore to modify LHRH pulse frequency and to adjust the level of homeostasis within the hypothalamus.

sensitivity. The critical tests for the duration hypothesis may be to conduct resonance and skeleton experimentsin pinealectomized animals but using melatonin infusions rather than light pulses. If animals could respond differentially to short and long durations given with a non-circadian periodicity this would be strong evidence for an "hour-glass" mechanism in the interpretation of melatonin rhythms.

The conclusions of the current studies are summarised in the model depicted in figure 6.1, thus the generation of melatonin rhythms depends on the entrainment by the light-dark cycle of two or more endogenous circadian oscillators, and the interpretation ultimately by the hypothalamus is a function of the duration of the peak.

Despite the strong evidence that melatonin rhythms produced by the pineal gland are central to photoperiodic responses in mammals, the site and mechanism (of action) of melatonin are still elusive, as evidenced by the paucity of information on this topic in several recent reviews (Goldman, 1983; Karsch et al., 1984; Tamarkin et al., 1985). Since the ultimate action of melatonin must be to influence LHRH secretion, it is possible that melatonin might directly influence opiatergic mechanisms. In mice, a diurnal rhythm in analgesia has been demonstrated which can be abolished by pinealectomy. Melatonin treatment can restore this nocturnal increase in analgesia in a naloxone reversible manner, thus indicating that the level of analgesia is related to EOP activity (Lakin et al., 1981). In aging mice, morphine is less potent at inducing analgesia. Pinealectomy can induce this "aging" effect in young mice, which might also indicate a link between pineal melatonin and central opiatergic mechanisms (Kavaliers et al., 1983). A circadian variation in met-enkephalin levels in the hypothalamus of the rat has been observed (Kumar et al., 1982) which is abolished by

pinealectomy, however few studies have been carried out on circadian aspects of EOP function. One study by Grossman et al. (1982b) failed to observe differential circadian effects of naloxone on ACTH release despite a pronounced underlying rhythm of ACTH secretion. The data on opioid analgesia in mice do not necessarily imply a direct effect of melatonin on opiatergic neurons, but the interaction between circadian melatonin rhythms and EOP functions within the hypothalamus would seem worthy of further study.

The studies described in chapters 3 and 4 provide good evidence that EOP mechanisms are involved in the central control of LH secretion in sheep. The actions of EOP are almost certainly on hypothalamic LHRH secretion rather than on pituitary LH release. Several studies have failed to demonstrate direct actions of EOP on the pituitary gland in vitro (Rivier et al., 1977; Grandison and Guidotti, 1977; Enjalbert et al., 1979), and in vivo opiates and opiate antagonists do not alter pituitary responsiveness to exogenous LHRH (Delitala et al., 1981; Owens and Cicero, 1981; Ferin et al., 1982). Certain problems arise in trying to extrapolate observations of plasma LH levels to hypothalamic secretion of LHRH. The amplitude of a plasma LH pulse does not directly indicate the amplitude of the hypothalamic LHRH pulse because changes in pituitary responsiveness to LHRH may modulate the hypothalamic signal. The studies in this thesis have concentrated on LH pulse frequency because in sheep LH pulses have never been observed without a corresponding hypothalamic LHRH pulse (Clarke and Cummins, 1982; Levine et al., 1982). The naloxone induced increases in LH pulse frequency observed in the current studies suggest that EOP mechanisms inhibit the LHRH pulse generator by modulating inputs to LHRH cell bodies. Some evidence exists in rats that EOP act on both adrenergic and

serotoninergic inputs to LHRH neurons (Kalra and Simpkins, 1981; Van Vugt et al., 1981b; Ieiri et al., 1980). In some situations increases in LH pulse amplitude were observed following naloxone treatment. It is not clear whether this represents an increased amplitude of LHRH release or an increased pituitary responsiveness due to the priming effects of a supraphysiological LHRH pulse frequency. Further studies using very frequent blood sampling techniques may clarify this question, but only direct measurement of LHRH in hypothalamic portal blood can provide unequivocal data.

The naloxone and morphine induced changes in LH pulse amplitude may indicate a direct neuromodulatory role of EOP on LHRH neurosecretion in the median eminence. Several lines of evidence for EOP actions on neurohormone release from neuron terminals exist. The current studies have demonstrated high levels of β-EP in this region, and immunocytochemical techniques also demonstrate EOP in the vicinity of LHRH neurons in the arcuate nucleus/median eminence region (Nilaver et al., 1979; Watson et al., 1978;). Opiate agonist and antagonist effects on LHRH and somatostatin release have been demonstrated using in vitro superfusion preparations of isolated mediobasal hypothalamus (Wilkes and Yen, 1981; Drouva et al., 1981). Opiate inhibition of oxytocin release from neurohumoral terminals in the posterior pituitary has been demonstrated (Clarke et al., 1979; Lincoln et al., 1985), and direct effects of opioids on dopamine release and turnover in the median eminence have also been observed (Ferland et al., 1977; Rotsztejn et al., 1978; Van Loon et al., 1980).

It is possible that the observed effects of naloxone on LH pulse frequency are actually effects on neurosecretion of LHRH. The studies by Clarke and Cummins (1982) and Levine et al. (1982) both demonstrated

that LHRH pulses of reduced amplitude occured in hypothalamic portal blood of castrate ewes which did not induce pulses of LH release from the pituitary. The effect of naloxone may be to block the opioid suppression which normally produces these "small" LHRH peaks, thus producing a sufficient signal for a pituitary response. This hypothesis can only be adequately tested by simultaneous measurement of portal blood LHRH and peripheral LH levels following naloxone, however it is of interest to note that at least on one occasion the LH pulse frequency in response to naloxone was greater than that observed even in castrate rams (see figure 3.6.23). Perhaps removal of opioid inhibition of LHRH release resulted in every "small" hypothalamic LHRH pulse being amplified to the level required for pituitary stimulation.

The initial interest in studying EOP mechanisms in sheep was to identify possible central mechanisms by which the long day inhibition of reproduction might be mediated. The original hypothesis was that EOP inhibition of LHRH release might induce sexual quiescence thus the opiate antagonist naloxone would have the greatest effect in stimulating LH release when rams were sexually regressed. The seemingly paradoxical result has been obtained that naloxone responses are greater when rams are sexually active, which implies that EOP inhibition on LHRH release is greatest when LHRH release itself is highest. Many studies in non-photoperiodic species have indicated a link between naloxone sensitivity and gonadal activity. Certain studies have shown that naloxone treatment does not increase plasma LH levels in long term castrate rats, though naloxone responses do persist for at least the first two days post gonadectomy (Bhanot and Wilkinson, 1983, 1984; Petraglia et al., 1984). This apparent loss of response maybe due to reduced pituitary responsiveness to LHRH in long term

castrates. The latter study used single LHRH injections to measure pituitary responsiveness, and although decreases were apparent the authors concluded that they were not sufficient to explain the loss of response to naloxone in castrate rats. Several studies have observed naloxone responses in castrate rats (Blank et al., 1980; Cicero et al., 1980, 1982). The variable results probably reflect the problem of single sampling in rats, subtle stimulatory effects of naloxone on pulsatile LH secretion in castrate rats may have been missed in the studies by Bhanot and Wilkinson (1983) and by Petraglia et al. (1984). One study in which serial blood samples were collected from castrate rats indicated subtle increases in LH pulse frequency and amplitude following naloxone treatment (Sylvester et al., 1982). There is , however, general agreement that administration of gonadal steroids restores or enhances the ability of naloxone to stimulate LH secretion in castrate rats (Cicero et al., 1979; Van Vugt et al., 1981a, 1982; Gabriel et al., 1983; Bhanot and Wilkinson, 1984; Petraglia et al., 1984), thus it has been hypothesised that EOP mechanisms mediate the negative feedback effects of gonadal steroids on the hypothalamus. The current finding that naloxone responsiveness in rams correlates with gonadal activity supports this hypothesis.

Two hypotheses might be proposed to explain the relationship between steroid feedback and the activity of inhibitory opiatergic mechanisms. The first is that gonadal steroids directly determine the level of EOP inhibition. The data obtained in the castrate and steroid implanted castrate rat model has generally been taken to support this hypothesis, and a second line of evidence is that ovariectomy of monkeys reduces levels of β-endorphin hypophyseal portal blood, and that steroid replacement reverses this decrease (Wardlaw et al., 1982a;

Wehrenberg et al., 1982). The lack of convincing evidence that naloxone responses are totally lost in castrate animals questions this hypothesis. Also changes in the level of opioid inhibition have been implicated in several normal physiological changes in the hypothalamo-pituitary-gonadal axis, for example in puberty, the menstrual cycle and the menopause. A more attractive hypothesis is that variation in opioid inhibition is a result of centrally induced changes and that steroid hormones are permissive in this system rather than directly causal. In sheep, therefore, the seasonal changes in apparent steroid feedback would be the result of centrally induced changes in the activity of EOP inhibitory mechanisms. The data from the pinealectomized rams indicate that photoperiod is not the only environmental cue capable of influencing EOP inhibition of LHRH secretion. Some preliminary data from another seasonally breeding mammal, the Syrian hamster, supports this "central" hypothesis. Naloxone responses in this species are very significantly reduced in hamsters transferred to inhibitory short days compared to those maintained on stimulatory long days (Eskes et al., 1984). Significant responses to naloxone cannot be shown in castrate hamsters kept on either photoperiod. However, testosterone implanted castrate hamsters do show an increase in LH levels when given naloxone on long days, though no response is seen in these steroid treated hamsters kept on short days (Swann and Turek, 1983). The implication is that photoperiod regulates the level of EOP inhibition, and interestingly in the hamster as in the sheep, this is highest when the animals are sexually active. The castrate and testosterone-implanted castrate ram would provide a good model to test the relationship between photoperiod, gonadal steroids, and EOP inhibition of LHRH release.

If the hypothesis is correct that the increase in LH secretion following naloxone treatment represents the degree of EOP inhibition on LHRH secretion, then the current studies and the studies in the Syrian hamster would suggest that steroid feedback has a greater suppressive effect on LHRH release during stimulatory photoperiods than during inhibitory photoperiods. In Soay rams at least, this implies that steroid feedback has a greater physiological role under short days as a homeostatic control mechanism within the hypothalamo-pituitary-gonadal axis than under long days as a mechanism by which photoperiod induces sexual quiescence. Testosterone feedback still plays an important homeostatic role in inhibiting pulsatile secretion under long days, though Soay rams castrated under long days show a slower increase in LH pulse frequency and amplitude and a lower steady state level than rams castrated under short days (Lincoln and Short, 1980). The observation that abrupt removal of gonadal steroids results in a more abrupt increase in LH secretion in rams maintained on short days may in itself be interpreted as evidence that there is a higher physiological level of gonadal steroid feedback under stimulatory photoperiod.

Figure 6.1 presents a model to explain the seasonal control of LHRH secretion which encompasses the findings of the current studies. The effect of photoperiod, other environmental cues, and the internal rhythm of reproductive activity is to both set the direct drive to the LHRH pulse generator and to accordingly adjust the level of negative steroid feedback which provides homeostatis within the H-P-G axis. The site of EOP inhibition may be on the actual neurosecretion of LHRH. Some experimental evidence exists for other aspects of the model. A recent study has demonstrated that pentobarbitol, a potent non-specific inhibitor of CNS function, transiently increases LH pulse frequency in

anoestrus ewes, indicating perhaps that long day photoperiods induce active inhibition on LHRH neurons (Goodman and Meyer, 1984). Studies with specific catecholamine antagonists indicate that this long day inhibition may be mediated by dopaminergic and noradrenergic neurons (Meyer and Goodman, 1983).

The current model resembles the steroid-dependent/steroid-independent model proposed by several authors (Turek and Ellis, 1981; Goodman and Karsch, 1981; Karsch et al., 1984) in that photoperiod and the other factors previously outlined appear to have a dual function in that they directly affect LHRH secretion, and they affect the level of steroid feedback on LHRH secretion. The crucial difference is in the relative empha sis placed on these two actions. In the previous model the principal effect of photoperiod is to alter the level of steroid feedback, thus steroid feedback is the mechanism by which photoperiod influences reproduction. The direct effect is necessarily a separate effect because it is defined in terms of the photoperiodic response seen in castrate animals where steroid effects have supposedly been eliminated. In the current model the principal effect of photoperiod is the direct control of LHRH secretion, and its role in modulating steroid feedback via opiatergic neurons is a mechanism to allow the H-P-G axis to function homeostatically at different levels of activity. It is quite possible in the current model that the supposed dual actions represent different aspects of the same mechanism.

The current studies attempted to investigate whether long term blockade of opioid inhibition might produce a functionally castrated ram. Chronic naloxone treatment was unsuccessful in this attempt because tolerance to its antagonist effects developed. Tolerance to the effects of opiate agonists is well described, but until recently

the phenomenon of tolerance to opiate antagonists was unknown (Owens and Cicero, 1981; Gabriel and Simpkins, 1983). Passive immunization against EOP in the brain might provide an alternative technique to study the effects of long term removal of opioid inhibition. In an acute study Schulz et al. (1981) have demonstrated that antisera to β-endorphin and to dynorphin increase LH levels in rats when injected into discrete hypothalamic areas, thus it appears that the technical problems of delivering antisera to an appropriate site of action can be overcome.

An interesting conjecture is that opiate tolerance and dependence may not just be pharmacological effects, but might be indicative of the actual physiological mechanisms by which EOP modulate neuroendocrine pathways (Lincoln et al., 1985). There is some evidence that central and peripheral opiatergic mechanisms adapt hibernating mammals to their low metabolic expenditure. Margules (1979) speculated that the gradual development of tolerance to these opiatergic effects might represent a mechanism by which the hibernation state is gradually reversed. It might equally be speculated that tolerance to the inhibitory effects of EOP provides a mechanism by which LHRH secretion increases when rams are becoming refractory to long days, and short day stimulation induces greater EOP release and consequently tolerance develops more rapidly.

In conclusion, the studies in this thesis have demonstrated that EOP mechanisms tonically inhibit LH release in rams, and suggest they may participate in the seasonal change in activity of the hypothalamic-pituitary-gonadal axis. These studies provide an exciting basis for future research, and perhaps provide a new direction for studies on the mechanism by which pineal melatonin rhythms influence reproductive function.

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